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Applicant: HARPOLD et al.

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Examiner: Ulm, J.

For: HUMAN NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR COMPOSITIONS AND METHODS EMPLOYING SAME

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Edwin C. Johnson, hereby declare as follows:

1) I received a Ph.D. in Biology from Purdue University, West Lafayette, Indiana, in 1984. Following receipt of the Ph.D., I worked at Brandeis University, Waltham, Massachusetts, as a postdoctoral Fellow from 1984-1987; and then at Marshall University School of Medicine, Huntington, West Virginia, initially as an Assistant Professor, and later, as an Associate Professor, from 1987-1992.

2) I am currently a Senior Research Scientist at SIBIA Neurosciences Inc., La Jolla, California (formally THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES INC., and hereinafter referred to as SIBIA). I have worked in the field of neurophysiology for at least 17 years. I am the author or co-author of more than 20 technical publications, primarily in the field of neuroscience.

3) In my capacity as a researcher at SIBIA, I have compared the functional properties of cloned nicotinic acetylcholine receptors (nAChR) from various species of animals. I have provided a DECLARATION executed January 27, 1995, demonstrating differences between rat and human nicotinic acetylcholine receptors.

4) A recent publication from SIBIA by Chavez-Noriega et al., entitled "Pharmacological Characterization of Recombinant Human Neuronal Nicotinic Acetylcholine Receptors h$\alpha 2\beta 2$, h$\alpha 2\beta 4$, h$\alpha 3\beta 2$, h$\alpha 3\beta 4$, h$\alpha 4\beta 2$, h$\alpha 4\beta 4$ and h$\alpha 7$ Expressed in Xenopus Oocytes" (J. Pharmacol. Exp. Ther., 280:346-356 (1997)), of which I am senior author, provides further evidence that human and rat nicotinic acetylcholine receptors (nAChR) have different pharmacological properties.

5) As described in Chavez-Noriega et al., recombinant human α and β nAChR subunits were expressed in xenopus oocytes to form active human nAChR and the oocytes were then evaluated for electrophysiological responses to various concentrations of nAChR agonists, including acetylcholine (AcCh), nicotine, cytisine (CYT) and 1,1-dimethyl-4-phenylpiperazinium (DMPP). The rank order potencies of the various agonists for the human nAChR was based on estimates of the EC_{50} from full dose-response curves. This was compared with literature values for rank order potency of recombinant rat nAChR that had been previously published by others. Comparison of the rank order of potency showed that human $\alpha3\beta2$, $\alpha3\beta4$ and $\alpha7$ nAChRs were different from rat $\alpha3\beta2$, $\alpha3\beta4$ and $\alpha7$ nAChRs(see e.g., Abstract and Discussion, page 353, 2nd Col. 2nd. full para.). Thus, these results further support the observation that the pharmacological properties of recombinant rat nAChR are not predictive of the cloned human nAChR. Full dose response curves for rat $\alpha2\beta2$ or $\alpha2\beta4$ nAChRs are not available in the literature, thereby precluding such comparison.

6) Figure 4 of Chavez-Noriega et al., shows partial dose response curves for each agonist with the particular human nAChR studied. The deduced rank order potency is based on a qualitative comparison of partial dose response curves for human $\alpha2\beta2$ and is stated to be similar to that published for the rat (see Discussion, page 353, 2nd Col. 2nd. full para.). This conclusion is not inconsistent with my prior statements regarding differences between these combinations of subunits and does not mean that rat and human $\alpha2\beta2$ are pharmacologically the same.

The prior DECLARATION provided data from a side-by-side comparison in which relatively low concentrations of the agonists were used. At other than low concnetrations of agonists, the receptors are subject to densitization and potential dampening of the response. In the experiments in the prior DECLARATION, $\alpha2\beta2$ and $\alpha3\beta2$ from human and rat were compared for rank order potency at the single low concentration of each agonist. These experiments demonstrated that $\alpha2\beta2$ of human and rat differed in rank order potency at the selected concentration.

To my knowledge, the comparison of pharmacological properties of recombinant human neuronal nicotinic acetylcholine receptors with those that of rat disclosed in my January 17, 1995 DECLARATION is the only quantitative side-by-side comparison performed in a manner that permits a meaningful comparison, whereby pharmacological differences could be observed.

In contrast, the experiments in Chavez-Noriega et al. with the human subunits were performed at SIBIA, but the data for the rat subunits was deduced from published reports. The comparisons from Chavez-Noriega et al. were based on a qualitative assessment of partial dose response curves.

Therefore, the results in the DECLARATION and the publication by Chavez-Noriega et al. demonstrate that functional properties of a human receptor cannot be predicted from the functional properties of a receptor from another species

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

5/9/97
Date

Edwin C. Johnson

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Comparative Structure of Human Neuronal α2–α7 and β2–β4 Nicotinic Acetylcholine Receptor Subunits and Functional Expression of the α2, α3, α4, α7, β2, and β4 Subunits

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Abstract

cDNA clones encoding human neuronal nicotinic acetylcholine receptor α2, α3, α4, α5, α6, α7, β2, β3, and β4 subunits were isolated from brainstem, hippocampus, prefrontal cortex, substantia nigra, thalamus, and IMR32 libraries. Human α2 and α6 and full-length β3 and β4 clones have not been previously reported. Deduced amino acid sequences of the α2, α6, β3, and β4 predicted mature peptides are 503 residues (56.9 kDa), 464 residues (53.7 kDa), 440 residues (50.8 kDa), and 477 residues (54.1 kDa), respectively. These sequences show 84 (α2), 87 (α6), 89 (β3), and 84% (β4) identity to the corresponding rat sequences. The amino termini of the human α2 and β3 mature peptides contain 23 and six additional residues, respectively, compared to those of rat α2 and β3. Recombinant receptors were expressed in *Xenopus laevis* oocytes injected with in vitro transcripts encoding either α7 alone or α2, α3, or α4 in pairwise combination with β2 or β4. Inward currents were elicited by the application of acetylcholine (1–100 μ*M*) and other agonists; these responses were blocked 65–97% by application of 10 μ*M* d-tubocurare, confirming functional expression of human nicotinic receptors.

Index Entries: Nicotinic acetylcholine receptor; molecular cloning; ligand-gated ion channel; central nervous system; human.

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels comprised of α and β subunits in a putative pentameric arrangement (Anand et al., 1991; Cooper et al., 1991). The proposed nAChR subunit structure consists of a long conserved amino-terminal extracellular domain followed by three highly conserved transmembrane domains (M1–M3), a variable cytoplasmic loop, a fourth conserved transmembrane domain (M4), and a short carb-

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oxyl terminal extracellular segment (Karlin, 1993). Two cysteines are conserved in the amino-terminal domain of all subunits, and two additional adjacent cysteines are conserved in the proposed ligand binding site of the α subunits. The neuronal nAChR subunits are encoded by a family of genes containing eleven known members. cDNA or genomic DNA clones have been isolated that encode subunits α2–α7 and β2–β4 (rat and chicken) (for review, *see* Sargent, 1993), α8 (chicken) (Schoepfer et al., 1990), and α9 (rat) (Elgoyhen et al., 1994). Full-length human α3, α4, α5, α7, and β2 clones have been reported, as well as partial human β3 and β4 clones (Anand and Lindstrom, 1990; Fornasari et al., 1990; Chini et al., 1992, 1994; Tarroni et al., 1992; Doucette-Stamm et al., 1993; Willoughby et al., 1993; Peng et al., 1994; Monteggia et al., 1995).

Functional recombinant rat and chicken neuronal nAChRs have been expressed in *Xenopus laevis* oocytes, composed of α2, α3, or α4 subunits in pairwise combination with β2 or β4 subunits (Boulter et al., 1987; Ballivet et al., 1988; Deneris et al., 1988; Wada et al., 1988; Couturier et al., 1990b). Rat and chicken α7 (Couturier et al., 1990a; Séguéla et al., 1993), chicken α8 (Gerzanich et al., 1994), and rat α9 (Elgoyhen et al., 1994) have been shown to be capable of forming functional homomeric receptors. Functional human α7 homomers have also been expressed in oocytes (Peng et al., 1994).

The subunit composition of heteromeric neuronal nAChRs influences their pharmacological properties. Sensitivity to agonists, such as acetylcholine (ACh), nicotine, cytisine, and 1,1-dimethyl-4-phenylpiperazinium (DMPP), in rat and chicken recombinant receptors varies for different subunit combinations (Couturier et al., 1990a; Luetje and Patrick, 1991; Séguéla et al., 1993; Elgoyhen et al., 1994). The pharmacological differences can be striking, such as the action of nicotine as an antagonist on homomeric rat α9 receptors in contrast to its agonist activity on most other nAChR subunit combinations (Elgoyhen et al., 1994).

Nicotinic receptor subunits also show species-specific differences in pharmacological properties. For example, the potency of nicotine, cytisine, and tetraethylammonium and both the potency and efficacy of DMPP differ significantly between chicken and human recombinant α7 homomers expressed in *Xenopus* oocytes, even though the subunits share 92% amino acid sequence identity (Peng et al., 1994).

Such species-specific differences in the pharmacology of nAChRs illustrate the importance of using human recombinant neuronal nAChR subunits in the development of therapeutics targeted at human neruonal nAChRs. Here, we report the isolation of cDNA clones and the assembly of full-length constructs containing the coding sequences for all of the known human neuronal nAChR subunits (α2–α7 and β2–β4) and present a comprehensive alignment of their deduced amino acid sequences. We compare the human α2, α6, β3, and β4 deduced amino acid sequences, reported for the first time in their entirety, with the homologous rat sequences. We demonstrate for the first time functional expression of recombinant human neuronal nAChR subunits in the pairwise combinations α2β2, α3β2, α4β2, α2β4, α3β4, and α4β4, and also show function for homo-oligomeric α7 receptors.

Experimental Procedures

cDNA Libraries

Human thalamus, prefrontal cortex, and hippocampus cDNA libraries were prepared essentially as described by Ellis et al. (1988). An IMR32 neuroblastoma cell line cDNA library was constructed as described in Williams et al. (1992). Human brainstem and substantia nigra cDNA libraries were obtained from the American Type Culture Collection (ATCC #37432) and Clontech Laboratories (Palo Alto, CA), respectively.

Probes and Hybridization Conditions

cDNA clones encoding rat nAChR subunits were generously provided by J. Boulter, S. Heinemann, J. Patrick, and colleagues (Salk Institute for Biological Studies, La Jolla, CA). Probes were prepared from rat α3 (nt –76–1782) (Boulter et al., 1986), α4–1 (nt –149–2078) (Goldman et al., 1987), α6 (nt –178–1582) (Boulter, J., unpublished results), α7 (nt –22–1873) (Boulter, J., unpublished results), β2 (nt –470–2016) (Deneris et al., 1988), and β4 (nt –86–1829) (Duvoisin et al., 1989) clones (nucleotides are numbered beginning with the adenine nucleotide in the initiation codon). A human α5 probe (nt

1108–1404) was amplified from IMR32 library DNA using PCR primers designed from a previously reported human α5 sequence (Chini et al., 1992). β3 subunit probes consisted of two synthetic oligonucleotides designed from the sequence of a partial human β3 cDNA clone (Willoughby et al., 1993) (nt 1–28 and nt 1240–1267 of the reported sequence). Approximately 1×10^6 recombinants were screened with ^{32}P-labeled probes following standard procedures (Maniatis et al., 1982). Hybridizations with cDNA fragment probes were performed in 50% deionized formamide, 5X Denhardt's solution, 5X SSPE, 0.2% SDS, and 200 μg/mL denatured sonicated herring sperm DNA for 16 h at 42°C. The formamide concentration was reduced to 10% for oligonucleotide hybridizations. Filters were washed at low (1X SSC, 0.2% SDS at 50°C), medium (0.2X SSPE, 0.2% SDS at 60°C), or high (0.1X SSPE, 0.1% SDS at 65°C) stringency, as noted below. Hybridizing plaques were identified by autoradiography and subsequently plaque purified.

Isolation of cDNA Clones

α2

Two overlapping partial human α2 clones were isolated from the thalamus library following low-stringency screening with the rat α4–1 probe. These clones were combined with a PCR fragment amplified from human thalamus cDNA to make a full-length α2 expression construct, KEα2RBS (nt 1–2112), as described in the following section.

α3

A partial human α3 clone was isolated from the brainstem library screened at low stringency with the rat α3 probe. This partial α3 clone was then used to screen the IMR32 library at high stringency, resulting in the isolation of the full-length human α3 clone KEα3.2 (nt –189–1720).

α4

A full-length human α4 clone, KEα4.2 (nt approx –350–3250), was isolated by screening the hippocampus cDNA library at medium stringency with the rat α4–1 probe.

α5

A full-length human α5 clone, KEα5.5 (nt –154–1674), was obtained following high-stringency screening of the IMR32 library with the human α5 PCR fragment described above.

α6

The human substantia nigra library was screened at medium stringency with the rat α6 probe, yielding full-length human α6 clone KEα6.3 (nt –142–1601).

α7

KEα7.3, a full-length human α7 clone (nt –72–1804), was isolated from the IMR32 library by medium-stringency screening with the rat α7 probe.

β2

A partial human β2 clone, isolated by low-stringency screening of the prefrontal cortex library with the rat β2 probe, was used to screen the thalamus library at high stringency. Two additional overlapping partial β2 clones were identified. A full-length β2 construct, hβ2.1F (nt –264–2184), was assembled by ligating adjacent restriction fragments of the three clones.

β3

The substantia nigra library was screened at medium stringency with the human β3 oligonucleotide probes described above, yielding a full-length human β3 clone, KBβ3.2 (nt –97–1830).

β4

KEβ4.6, a full-length human β4 clone (nt –86–1829), was isolated from the IMR32 library by medium-stringency screening with the rat β4 probe.

Sequence Analysis

cDNA inserts were sequenced with an ABI Model 373A DNA Sequencing System (Applied Biosystems, Foster City, CA). The University of Wisconsin Genetic Computer Group and DNASTAR (Madison, WI) software packages were used for data analysis. The cDNA sequences were deposited in GenBank, accession numbers U62431–U62439.

Functional Expression of Human Subunits

The following modifications were performed for the nAChR subunit expression studies reported here.

α2

The α2 5' untranslated region (UTR) was replaced with a Kozak consensus ribosomal binding site (RBS), 5'-GCCACC-3' (Kozak, 1987) during amplification of the 5' end of the coding sequence from human thalamus cDNA by polymerase chain reaction (PCR). The sense primer consisted of an adaptor segment containing appropriate restriction sites plus the RBS adjacent to a segment specific for nt 1–24 of the coding sequence, and the antisense primer was located 3' to a convenient restriction site. A restriction fragment of the PCR product was ligated with adjacent fragments of the two partial α2 clones described above to construct KEα2RBS (nt 1–2112).

α3

Construct KEα3 (nt –12–1720) was generated by removing the majority of the 5' UTR from KEα3.2 by a restriction digest and religation.

α4 and α7

The 5' UTRs of KEα4.2 and KEα7.3 were replaced with the Kozak consensus RBS by amplifying modified 5' ends of the coding sequences from the cDNA clones and ligating with appropriate restriction fragments as described above, yielding constructs KEα4RBS (nt 1–3250) and KEα7RBS (nt 1–1804).

β2

The 5' UTR of hβ2.1F was also replaced with the RBS as described above to generate construct KEβ2RBS (nt 1–2184). In addition, a chimera was constructed in which the rat 5' UTR and signal peptide coding sequences were linked to the mature human β2 peptide coding sequence (nt –179–120 from rat β2 clone PCX49(1) and nt 112–1509 from hβ2.1F), yielding construct pSP65–rhβ2.

β4

KEβ4.6 was not modified for expression. The cDNAs were subcloned into plasmid expression vectors as follows: α2, α3, and β4 in pCMVβ (Clontech Laboratories, Palo Alto, CA), modified by the insertion of a T7 promoter; α4 in pIBI24 (International Biotechnologies, New Haven, CT); α7 in pBSTA (Goldin and Sumikawa, 1992); β2 in pSP64T (Krieg and Melton, 1984); and the chimeric β2 construct in pSP65 (Promega, Madison, WI). In vitro transcripts were prepared using MegaScript T7 or SP6 capped RNA transcription kits (Ambion, Austin, TX).

Stage V oocytes were isolated from *Xenopus laevis* using standard techniques (Goldin and Sumikawa, 1992). Oocytes were typically injected with 10–50 ng of one or two in vitro transcripts. Two to six days later, the oocytes were voltage-clamped (OC-725B, Warner Instruments [Hamden, CT], Geneclamp, Axon Instruments [Foster City, CA]) with two microelectrodes and held at –80 mV in a bath containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES at pH 7.3, and 0.001 mM atropine. Drugs were applied by gravity feed into a recording chamber (Warner Instruments). Data were filtered at 10 Hz and digitized at 100 Hz. Data collection and analysis used Axotape and pClamp software (Axon Instruments).

Results and Discussion

Comparison of Human and Rat Sequences

cDNA clones encoding the human neuronal nAChR α2, α3, α4, α5, α6, α7, β2, β3, and β4 subunits were isolated and constructs containing the complete coding sequences were prepared as described in Experimental Procedures. The isolation of α2 clones from a human thalamus cDNA library is of interest, since α2 mRNA was not detected in rat thalamus by *in situ* hybridization (Wada et al., 1989). This may reflect a difference between human and rat in the distribution of mRNA encoding the α2 subunit, or it could be explained by the presence of nonthalamic tissue in the human tissue sample from which the cDNA was prepared.

Full-length human α2, α6, β3, and β4 cDNA clones are reported for the first time. The deduced amino acid sequences of these clones are given in Fig. 1 (*see* pp. 222,223) aligned with the homologous rat sequences. The β3 deduced amino acid sequence contains 35 additional residues at the amino terminus compared to the partial sequence reported by Willoughby et al. (1993), and the sequences also differ at the first three residues of the region of overlap and one residue in the fourth transmembrane domain. The β4 deduced amino acid sequence contains 47 additional amino terminal residues compared with the

sequence reported by Tarroni et al. (1992) and differs at two residues in the amino-terminal extracellular domain. Overall, the human and rat α2, α6, β3, and β4 mature peptide sequences are 84, 87, 89, and 84% identical, respectively. The amino termini of the human α2 and β3 mature peptides differ from those of the rat, containing an additional 23 and six residues, respectively.

The deduced amino acid sequences of the human α2–α7 and β2–β4 clones isolated in this study are given in Fig. 2 (*see* p. 224). The α4 and β2 sequences are identical to previously reported sequences (Doucette-Stamm et al., 1993; Monteggia et al., 1995). The α7 sequence differs from the consensus of the three published human α7 sequences (Anand and Lindstrom, 1990; Chini et al., 1994; Peng et al., 1994) at one residue in the signal peptide. The human α3 nucleotide sequence differs from that reported by Fornasari et al. (1990) at several sites. Six separate nucleotide insertions near the 5' end of the coding sequence change the amino terminus of the signal peptide from MALAV to MGSGPL, and six nucleotides in the amino-terminal extracellular and cytoplasmic domains differ from the above sequence but agree with that of a partial human α3 clone in GenBank (accession #X53559). The nucleotide sequence of the human α5 clone shows two deletions and two insertions compared to the α5 coding sequence reported by Chini et al. (1992), changing the amino terminus of the putative mature peptide from ALRSSRARRAAR to RCGLAGAAGGAQ.

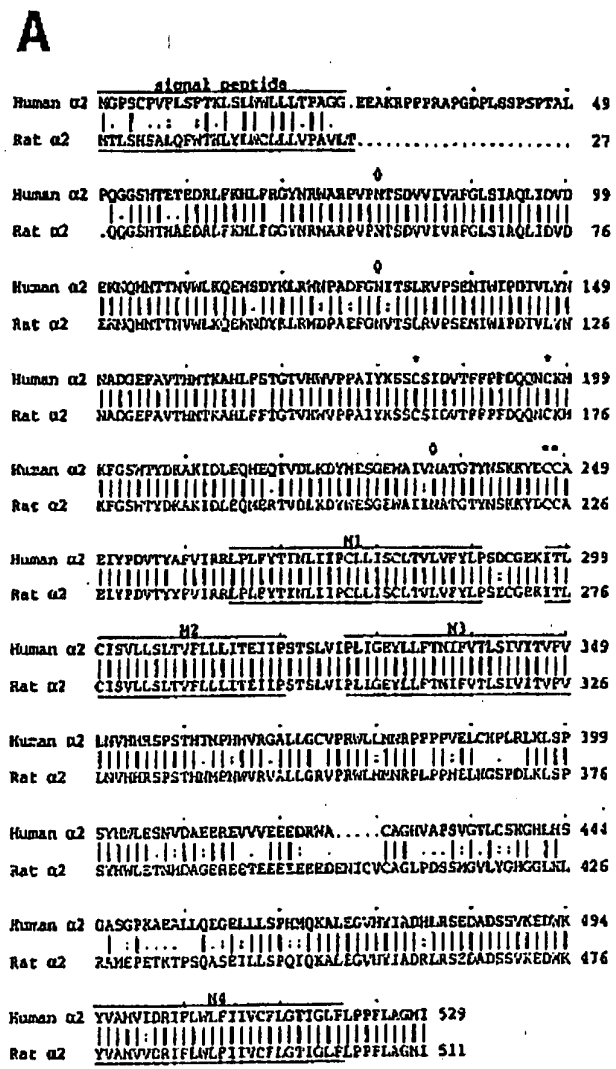
The deduced amino acid sequences of the putative major structural domains of human and rat subunits are compared in Table 1. The amino-terminal extracellular region and four transmembrane domains are highly conserved (91–99% and 86–100% identity, respectively). The identity between human and rat sequences in the M2 region thought to form the pore of the ion channel (Karlin, 1993) is 96–100%. Two regions show greater divergence between human and rat, the cytoplasmic domain (67–89% identity) and the signal peptide (27–86% identity).

This pattern of conservation of residues in the amino-terminal and transmembrane domains and sequence divergence across the cytoplasmic domains and signal peptides is also seen across subunits within a species. Figure 2 illustrates the relationship among the nine human neuronal nAChR α and β subunits. The cysteines characteristic of nAChR subunit amino-terminal extracellular domains are conserved. Eight residues believed to participate in the ACh binding site have been identified in the *Torpedo* α subunit by photoaffinity labeling: Trp^{86}, Tyr^{93}, Trp^{149}, Tyr^{151}, Tyr^{190}, Cys^{192}, Cys^{193}, and Tyr^{198} (Dennis et al., 1988; Galzi et al., 1990). These residues are all conserved in the human α2, α3, α4, α6, and α7 subunits. In contrast, the human and rat α5 sequences (Boulter et al., 1990) differ from the *Torpedo* α sequence at two of these sites: Tyr^{93} is replaced by Phe and Tyr^{190} is replaced by Asp. Site-directed mutagenesis studies in the chicken α7 subunit have shown that substitutions at these two sites can cause a fivefold or greater decrease in receptor sensitivity to ACh (Galzi et al., 1991).

Expression of Functional Receptors

Functional expression of recombinant human neuronal nAChRs was examined in *Xenopus* oocytes for the subunit combinations α2β2, α3β2, α4β2, α2β4, α3β4, α4β4, and α7 alone. Following injection of in vitro transcripts prepared from cDNAs encoding the nAChR subunits, responses were elicited by superfusion of ACh and recorded with two-electrode voltage clamp. For each subtype combination, inward currents were observed during the application of 0.3–100 μ*M* ACh to oocytes voltage clamped at –80 mV. Figure 3 shows the response of each nicotinic subtype combination to ACh. Inward currents in response to cytisine, nicotine, DMPP, and methylcarbamylcholine were also detected (data not shown). Oocytes injected with in vitro transcripts encoding α2, α3, α4, β2, or β4 alone did not respond to nicotinic agonists (data not shown); however, inward currents were observed in oocytes injected with α7 transcripts alone.

Inhibition of nicotinic responses in oocytes injected with in vitro transcripts of human nAChR subunits by the nAChR antagonist d-tubocurare is summarized in Table 2. A nonsaturating dose of a potent agonist was chosen for each subtype. The agonists were applied in the absence and presence of 10 μ*M* d-tubocurare. Human nAChRs with the subunit combinations α2β4, α3β2, α3β4, α4β4, and α7 alone are the most sensitive to block by d-tubocurare. Human α2β2 and α4β2 nAChRs


A
signal peptide
Human α2 MGPSCPVPLSPTKLSLIWLLLTPAGG.EEAKRPPFRAPGDPLGSPSPTAL 49
Rat α2 MTLSHSALQFWTKLYIWCLLLVPAVLT..... 27
Human α2 PQGGSHTETEDRLFKHLFRGYNRWARPVPNTSDVVIVAFGLSIAQLIDVD 99
Rat α2 .QGGSHTHAEDALFKHLFGGYNRWARPVPNTSDVVIVAFGLSIAQLIDVD 76
Human α2 EKNQMNTTNVWLKQEWSDYKLRWNPADFGNITSLRVPSENIWIPDIVLYN 149
Rat α2 EKNQMNTTHVWLKQEWNDYRLRWDPAEFGNVTSLRVPSEMIWIPDIVLYN 126
Human α2 NADGEFAVTHMTKAHLFSTGTVHWVPPAIYKSSCSIDVTFFPFDQQNCKM 199
Rat α2 NADGEFAVTHMTKAHLFFTGTVHWVPPAIYKSSCSIDVTFFPFDQQNCKM 176
Human α2 KFGSWTYDKAKIDLEQMEMTVDLKDYWESGEWAIVNATGTYNSKKYECCA 249
Rat α2 KFGSWTYDKAKIDLEQMERTVDLKDYWESGEWAIRNATGTYNSKKYLCCA 226
Human α2 EIYPDVTYAFVIARLPLFYTINLIIPCLLISCLTVLVFYLPSDCGEKITL 299
Rat α2 EIYPDVTYKFVIARLPLFYTINLIIPCLLISCLTVLVFYLPSECGEKITL 276
Human α2 CISVLLSLTVFLLLITEIIPSTSLVIPLIGEYLLFTMIFVTLSIVITVFV 349
Rat α2 CISVLLSLTVFLLLITEIIPSTSLVIPLIGEYLLFTMIFVTLSIVITVFV 326
Human α2 LNVHHRSPSTHTMPHHVRGALLGCVPRWLLMKRPPPPVELCKPLRLKLSP 399
Rat α2 LNVHHRSPSTHTMPHHVRVALLGRVPRWLHMNRPLPPHELKGSPDLKLSP 376
Human α2 SVIGALESNVDAEERVVVEEEDRNA.....CAGHVAFSVGTLCSKGHLHS 444
Rat α2 SKHWLETNMDAGERECTEEEELEEEDENICVCAGLPDSSMGVLYGKGLKL 426
Human α2 QASGPKAEALIQEGELLLSPHMQKALEGVHYIADHLRSEDADSSVKEDWK 494
Rat α2 RAMEPETKTPSQASELILLSPQIQKALEGVHYIADRLRSEDADSSVKEDWK 476
Human α2 YVAMVIDRIFLWLFIIVCFLGTIGLFLPPFLAGMI 529
Rat α2 YVAMVVGRIFLWLPIIVCFLGTIGLFLPPFLAGMI 511
B
signal peptide
Human α6 MLTSKGQGFLHGGLCLMLCVFTPFFRGCVGCATEERLFHKLFSHYNQFIR 50
Rat α6 MLWGHGAGDLRSGLCLMICGPLAPFRGSRGCVSEEQLFHTLFAHYNRFIR 50
Human α6 PVENVSDPVTVHFEVAITQLANVDEVNQIMETNLWLRHVWNDYKLRWDPN 100
Rat α6 PVENVSDPVTVHFELAITQLANVDEVNQIMETNLWLRHVWKDYRLCWDPT 100
Human α6 EYDGIETLRVPADKIWRPDIVLYNNAVGDFQVEGKTKALLKYTGHITWTP 150
Rat α6 EYDGIETLRVPADHIWKPDIVLYNNAVGDFQVEGKTKALLKYDGVITWTP 150
Human α6 PAIFKSSCPNDITFFPFDWQNCSLKFGSWTYDKAEIDLIIIGSKVEMNDF 200
Rat α6 PAIFKSSCFNDI?FFPFDWQNCSLKFGSWTYDKAEIDLIIIGSKVEMNDF 200
Human α6 WENSEWEIIDASGYKMDIKYNCCEEIYTDITYSFYIRRLPMFYTINLIIP 250
Rat α6 WENSEWEIVDASGYKMDIKYNCCEEIYTDITYSFYIRRLPMFYTINLIIP 250
Human α6 CLFIGFLTVLVFYLPSDCGEKVTLCISVLLSLTVFLLVITETIPSTSLVV 300
Rat α6 CLFISFLTVLVFYLPSDCGEKVTLCISVLLSLTVFLLVITETIPSTSLVI 300
Human α6 PLVGEYLLFTMIFVTLSIVVTVFVLNIHYRTPTTHTMPRWVKTVFLKLLP 350
Rat α6 PLVGEYLLFTMIFVTLSIVVTVFVLNIHYRTPATHTMPKWVKTMFLQVFP 350
Human α6 QVLLMRWPLDKTRGTGSDAVPRGLARRPARGKLASKGRPRHLKECTHCHK 400
Rat α6 SILMDPPLDKTKEMDGVKQPKTHTKRPAKVKFTHRKEPKELKECAHCHK 400
Human α6 SNELATSKRPLSHQPLQWVVENSEHSPEVEDVINSVQPIAENMKSIDIETK 450
Rat α6 SSEIAPGK.RLSQQPAQWVTENSEHPPDVEDVIDSVQFIAENMKSIHIETK 449
Human α6 EVEDDWKYVAHVVDRVFLWVFIIVCVTGTAGLFLQPLLGNTGKS 494
Rat α6 EVEDGWKYMANVVDRVFLWVPIIVCVFGTVGLFLQPLLGNTGAS 493

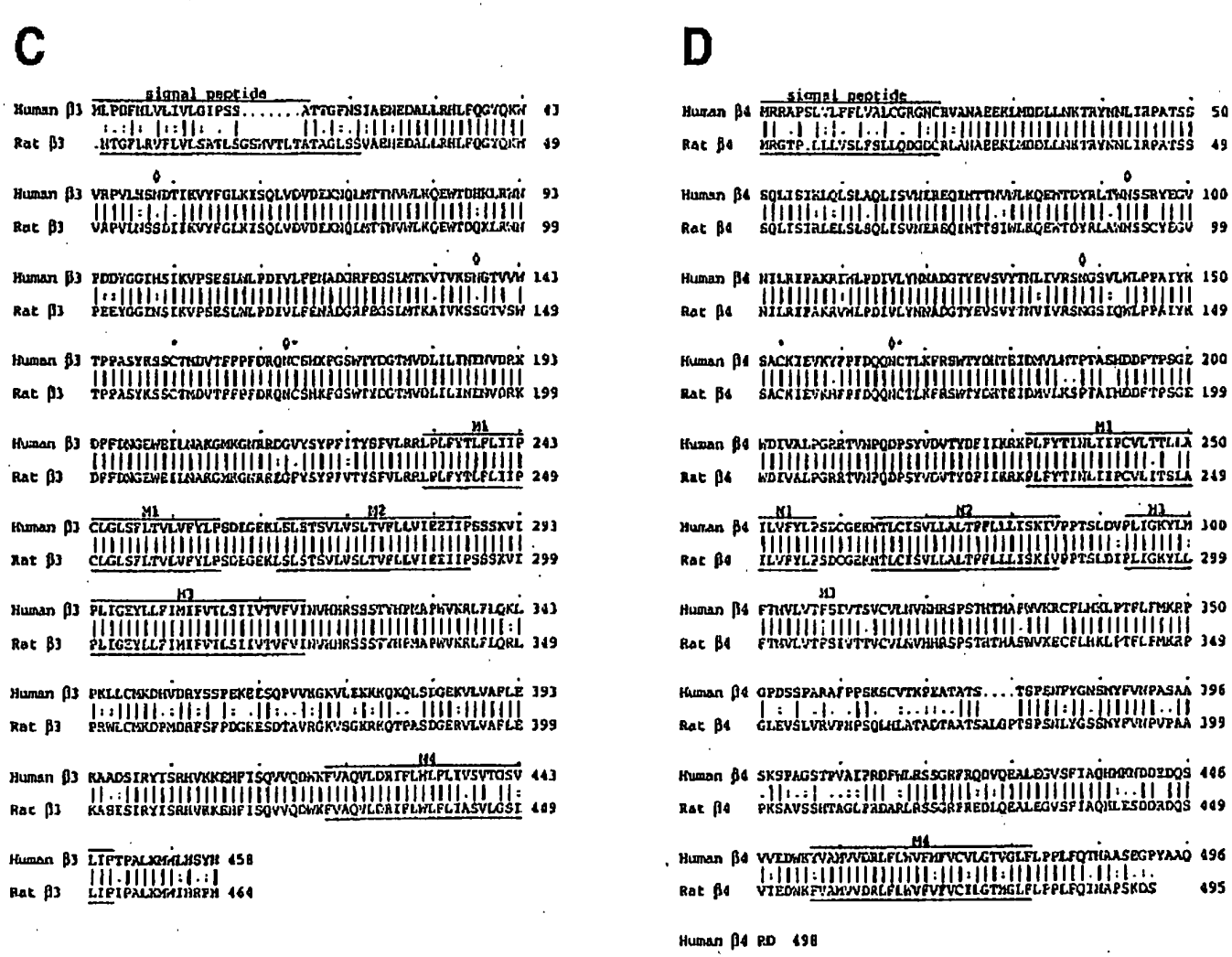


Fig. 1. Comparisons of the deduced amino acid sequences of human and rat α2, α6, β3, and β4 nAChR subunits. The residues are numbered beginning with the initiating methionine. Putative signal peptide and transmembrane domains (M1–M4), conserved cysteine residues (*), and potential N-linked glycosylation sites (◇) are indicated. The signal peptide cleavage sites were predicted according to von Heijne (1986). Molecular weights are given for the nonglycosylated mature peptides. (A) Clone KEα2RBS contains a 1587-bp open reading frame encoding 529 amino acids with a predicted mature peptide of 503 residues (56.9 kDa) showing 84% identity with that of rat α2. (B) Clone KEα6.3 has an open reading frame of 1485 bp that encodes 494 amino acids. The 464-residue (53.7 kDa) predicted mature peptide shows 87% identity with that of rat α6. (C) Clone KBβ3.2 contains a 1377-bp open reading frame encoding 458 amino acids and a putative mature peptide of 440 residues (50.8 kDa) with 89% identity to that of rat β3. An alternative possible signal peptide cleavage site, scoring slightly lower by the rules of von Heijne, is located following T^{20}. (D) Clone KEβ4.6 contains a 1494-bp open reading frame encoding 498 amino acids with a predicted mature peptide of 477 residues (54.1 kDa) showing 84% identity with that of rat β4.

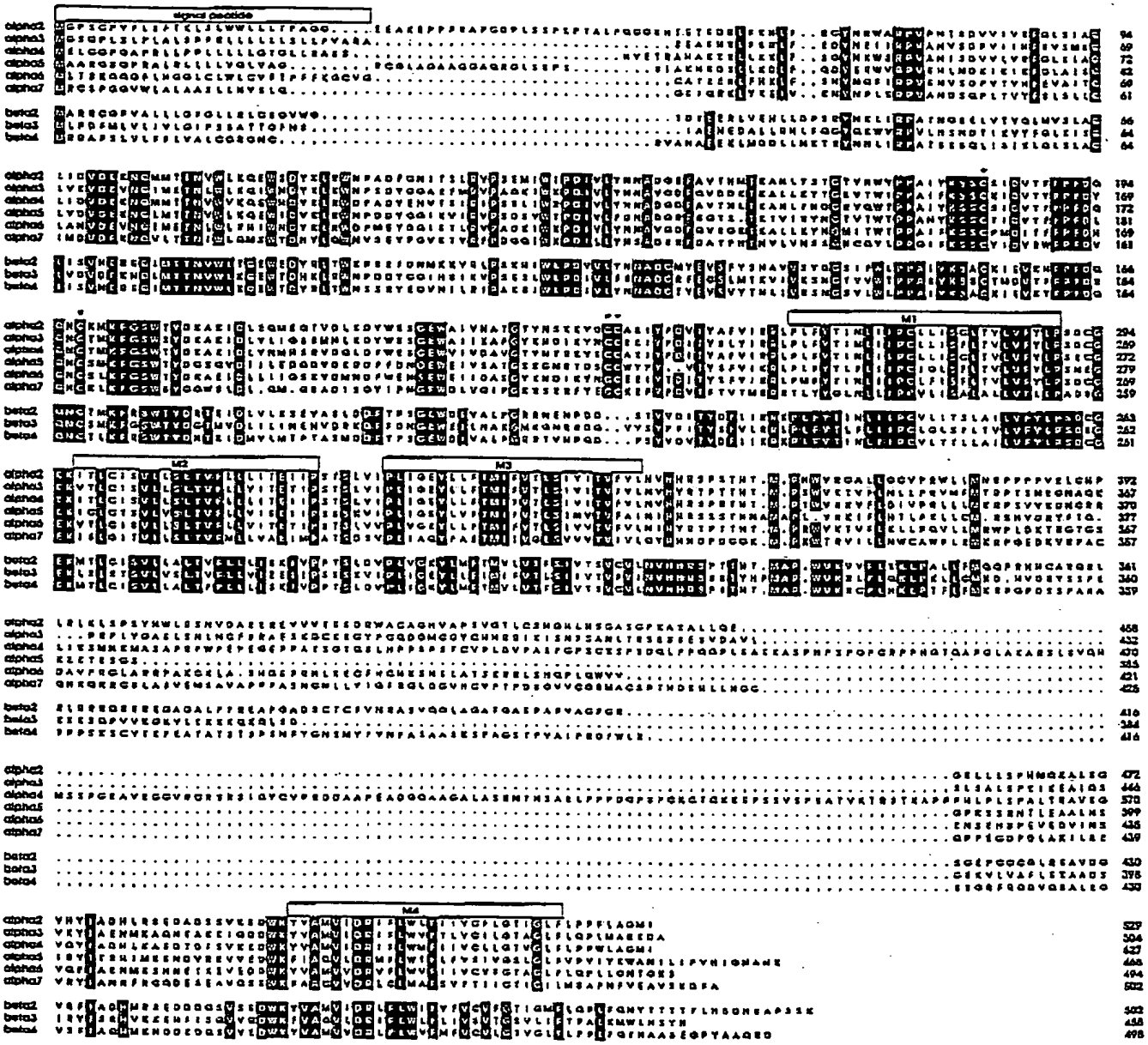


Fig. 2. Alignment of deduced amino acid sequences of human neuronal nAChR subunits. An alignment of human nAChR amino acid sequences is presented for subunits α2, α3, α4, α5, α6, α7, β2, β3, and β4. Residues conserved in all α or all β subunits are highlighted. Dots represent gaps introduced to optimize the alignments. Conserved cysteines (*), putative signal peptides, and transmembrane domains (M1–M4) are indicated.

Table 1
Comparison of Human and Rat nAChR Deduced Amino Acid Sequences[a]

Putative domain	α2	α3	α4	α5	α6	α7	β2	β3	β4
Signal peptide	42	40	85	27	57	73	54	28	35
Mature peptide	84	91	86	92	82	91	95	89	84
Amino-terminal	94	93	97	93	92	94	99	92	91
M1	100	100	100	96	100	100	100	100	92
M2	100	100	96	96	100	100	100	100	100
M3	100	96	100	100	100	86	100	100	92
Cytoplasmic	67	89	69	86	68	82	88	77	68
M4	95	96	100	88	92	100	96	88	85

[a]Percent identity between rat and human primary sequences for each subunit in the major putative structural domains. The calculations were performed on regions present in both sequences.

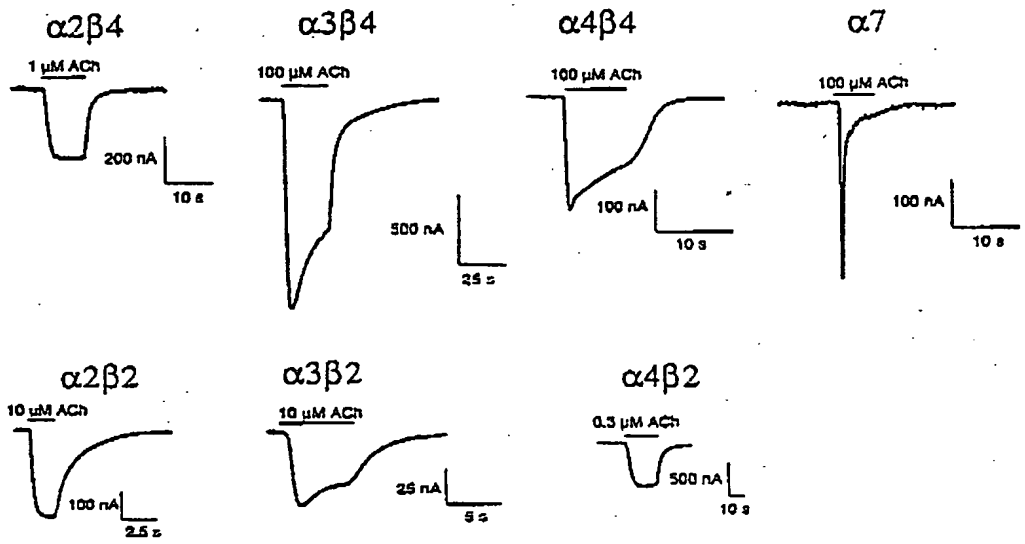


Fig. 3. Acetylcholine-induced currents in recombinant human neuronal nAChRs expressed in *Xenopus laevis* oocytes. Oocytes were injected with in vitro transcripts prepared from constructs KEα2RBS, KEα3, KEα4RBS, KEα7RBS, pSP65-rhβ2, and KEβ4.6. Oocytes expressing human nAChR subunits were voltage-clamped at a holding potential of –80 mV and inward currents were elicited by application of ACh (0.3–100 μM). Seven subunit combinations produced functional nAChRs: α2β4; α3β4; α4β4; α7; α2β2; α3β2; α4β2.

appear less sensitive to d-tubocurare. Further concentration-response studies are needed to confirm this. The sensitivity to d-tubocurare of responses elicited in oocytes injected with human α7 transcripts is similar to those reported for human (Peng et al., 1994), rat (Séguéla et al., 1993), and chicken (Bertrand et al., 1992) α7 nAChRs, and the block in oocytes injected with the α4β2 combination is similar to that reported for chicken α4β2 nAChRs stably expressed in mouse fibroblast cells (Whiting et al., 1991). Our observation that all of the tested subunit combinations are sensitive to block by d-tubocurare supports the view that these currents are elicited by the activation of nicotinic receptors.

Table 2
Inhibition of nAChR Agonist-Induced Responses by D-Tubocurare[a]

Subtype	Agonist	Percent of control
α2β2	Nicotine (1 μM)	23 ± 15
α2β4	ACh (30 μM)	9 ± 8
α3β2	DMPP (10 μM)	6 ± 4
α3β4	DMPP (10 μM)	10 ± 5
α4β2	ACh (10 μM)	35 ± 4
α4β4	Nicotine (10 μM)	4 ± 2
α7	ACh (100 μM)	3 ± 1

[a]Nicotinic agonists were applied in the absence and presence of d-tubocurare (10 μM) to oocytes expressing human nAChRs. Percent of control indicates the amplitude of the response elicited by each agonist in the presence of the antagonist as a percent of the amplitude of the response to that agonist in the absence of antagonist (mean ± SD; n = 3–10 oocytes/group).

The cloning and expression of human neuronal nAChR subunit cDNAs present new opportunities to advance our understanding of the role of nicotinic receptors in the central nervous system. The human α2–α7 and β2–β4 cDNA clones provide specific probes for mapping the distribution of human subunit mRNAs. A complete set of human subunit-specific antibodies can now be generated in order to study the composition and distribution of native human neuronal nAChR subtypes in tissue slices and cultured cells. We are in the process of characterizing functional expression of the human α2, α3, α4, α7, β2, and β4 subunit cDNAs, and expression studies with the human α5, α6, and β3 clones and various multimeric combinations are in progress. The use of human clones is important for elucidating the function and properties of human neuronal nicotinic receptors, since the alteration of even a single residue in a subunit can affect the pharmacological properties of a receptor (Hussy et al., 1994). The functional expression of human neuronal nAChR cDNAs will allow us to investigate differences in pharmacological properties between human neuronal nAChR subtypes and will facilitate the identification of compounds useful in the prevention or treatment of neurological diseases involving nicotinic receptors.

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Pharmacological Characterization of Recombinant Human Neuronal Nicotinic Acetylcholine Receptors hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 Expressed in *Xenopus* Oocytes

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ABSTRACT

Human neuronal nicotinic acetylcholine receptors (nAChRs) hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 were expressed in *Xenopus* oocytes and tested for their sensitivities to the nicotinic agonists acetylcholine (ACh), nicotine, cytisine (CYT) and 1,1-dimethyl-4-phenylpiperazinium (DMPP) and the nAChR antagonists mecamylamine (MEC), d-tubocurarine and dihydro-β-erythroidine. CYT was the least efficacious agonist at hnAChRs containing β2 subunits, but it displayed significant activity at hα2β4, hα3β4, hα4β4 and hα7 nAChRs. ACh was one of the most efficacious agonists at all hnAChRs, except at hα3β2, where DMPP was markedly more efficacious than ACh. ACh was among the least potent agonists at all hnAChRs. The rank order of potency displayed by hα3β2 and hα3β4 nAChRs (DMPP≈CYT≈nicotine>ACh and DMPP > CYT≈nicotine>ACh, respectively), differs from that reported for their rat homologs (Luetje and Patrick, 1991; Covernton *et al.*, 1994). The agonist profile observed in hα7 also differs from that reported for its rat homolog (Seguela *et al.*, 1993). Human α4β2 and hα4β4 nAChRs were more sensitive to dihydro-β-erythroidine than d-tubocurarine, whereas hα7 and hα3β4 were more sensitive to d-tubocurarine than dihydro-β-erythroidine. These antagonists were equipotent at hα2β2, hα3β2 and hα2β4 nAChRs. MEC (3 μM) inhibited hα2β4 and hα4β4 nAChRs by > 80%, whereas hα2β2, hα4β2 and hα7 nAChRs were inhibited by approximately 50%. Taken together, the differential sensitivities observed at various recombinant hnAChR subtypes indicate that both α and β subunits contribute to the pharmacology of these ligand-gated channels. The unique selectivity profiles displayed by human nAChRs constitute a valuable tool for the development of selective nicotinic analogs as potential therapeutic drugs.

nAChRs are ligand-gated ion channels activated by the neurotransmitter ACh and are distributed throughout the peripheral and central nervous system (Clarke *et al.*, 1985; Wada *et al.*, 1989; Dineley-Miller and Patrick, 1992; Séguéla *et al.*, 1993; Rubboli *et al.*, 1994). To date, a gene family encoding 11 nAChR subunits has been identified (Elgoyhen *et al.*, 1994; for a review see Sargent, 1993). We and others have cloned nine human nAChR subunits: α2, α3, α4, α5, α6, α7, β2, β3 and β4 (Elliott *et al.*, 1996; Fornasari *et al.*, 1990; Chini *et al.*, 1992; Anand and Lindstrom, 1990; Tarroni *et al.*, 1992; Doucette-Stamm *et al.*, 1993; Peng *et al.*, 1994; Willoughby *et al.*, 1993). The stoichiometry of recombinant nAChRs expressed in *Xenopus* oocytes is thought to be $(\alpha x)_2(\beta y)_3$ (Anand *et al.*, 1991; Cooper *et al.*, 1991); however, in recombinant expression systems α7, as well as α8 and α9 can form functional homooligomeric receptors (Couturier *et al.*, 1990; Gerzanich *et al.*, 1994; Elgoyhen *et al.*, 1994).

Pharmacological and functional studies of recombinant rat and chicken nAChRs expressed in *Xenopus* oocytes have revealed a large diversity among the different subunit combinations (Luetje and Patrick, 1991; Connolly *et al.*, 1992; see Sargent, 1993 and Papke, 1993 for review). Recent reports on the functional characterization of hα7 (Peng *et al.*, 1994; Gopalakrishnan *et al.*, 1995) and hα4β2 nAChRs (Gopalakrishnan *et al.*, 1996) indicate that the human homologs are also pharmacologically and functionally diverse.

Many different subtypes of nAChRs have been reported in a variety of neurons; nAChRs are present in both pre- and postsynaptic structures in the rodent and chick central nervous system (reviewed by Sargent, 1993 and Clarke, 1995).

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ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptors; ACh, acetylcholine; NIC, (-)nicotine; CYT, cytisine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; MEC, mecamylamine; d-Tubo, d-tubocurarine; DHβE, dihydro-β-erythroidine; DRCs, dose-response curves; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid].

One approach to gain insight into the molecular composition of native nAChRs has been to compare their functional and pharmacological profiles with those observed using recombinant receptors. The molecular composition of some native chick and rat nAChRs has been proposed based on their pharmacological profile and the characteristics of their macroscopic currents (Mulle *et al.*, 1991; Alkondon and Albuquerque, 1993; Covernton *et al.*, 1994; Zhang *et al.*, 1994). However, at the single-channel level, a good correlation has not yet been established between native and recombinant nAChRs tested to date (Connolly *et al.*, 1995, for reviews see McGehee and Role, 1995; Sargent, 1993 and Papke, 1993).

Administration of nicotinic agonists to rodents increases locomotor activity and enhances learning and memory, as shown in several behavioral tests (Clarke and Kumar, 1983; Levin *et al.*, 1993). In human neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, there is a significant reduction in nAChR number (Rinne *et al.*, 1991; Nordberg, 1994), and administration of nAChR agonists may ameliorate many of the motor and cognitive deficits associated with these diseases (Baron, 1986; Newhouse *et al.*, 1988) and other motor disorders, such as Tourette's syndrome (Moss *et al.*, 1989). More recently, a missense mutation in the hnAChR subunit $\alpha 4$ was found to be associated with a form of familial frontal lobe epilepsy (Steinlein *et al.*, 1995). Identification and characterization of the hnAChR subtypes involved in these phenomena may therefore be critical in the development of subtype-specific nAChR modulators for therapeutic purposes.

Human nAChR subunits $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$ show 91–99% amino acid identity to their rat homologs in their extracellular amino terminal domain (Anand and Lindstrom, 1990; Fornasari *et al.*, 1990; Chini *et al.*, 1992; Doucette-Stamm *et al.*, 1993; Willoughby *et al.*, 1993; Peng *et al.*, 1994; Elliott *et al.*, 1996). These differences in the deduced amino acid sequences may affect the properties of nAChRs: substitution of a single amino acid residue in the extracellular amino terminal region of $\alpha 3$ (Hussy *et al.*, 1994) and $\alpha 7$ (Galzi *et al.*, 1991) nAChRs subunits has been shown to dramatically affect the pharmacology of recombinant nAChRs. Studying the properties of hnAChRs using heterologous expression may therefore provide valuable insights into the composition, function and pharmacology of native hnAChRs. We now report that, when expressed in *Xenopus* oocytes, recombinant hnAChRs display unique sensitivities to nAChR agonists and antagonists, and the pharmacology of some of these hnAChRs differs from that reported for their rat homologs.

Methods

Clones. The hnAChR subunits $\alpha 2$, $\alpha 3$, $\alpha 4$-2, $\beta 2$, $\beta 4$ and $\alpha 7$ were cloned from cDNA libraries prepared from human brain and the human IMR32 neuroblastoma cell line (Elliott *et al.*, 1996). GenBank access numbers for the cDNA nucleotide sequences are U62431-U62439 ($\alpha 2$-$\alpha 7$ and $\beta 2$-$\beta 4$, respectively). The 5' untranslated region of $\alpha 2$, $\alpha 4$-2, $\beta 2$ and $\alpha 7$ cDNA was removed and replaced with a Kozak consensus ribosomal binding site, 5'-GCCACC-3' (Kozak, 1987). The cDNAs were subcloned into different expression vectors, as indicated in Elliott *et al.* (1996), except that the KEβ2RBS insert was subcloned into a pCMV vector modified by the insertion of a T7 promoter. *In vitro* transcripts were prepared using MegaScript T7 or SP6 capped RNA transcription kits (Ambion, Inc., Austin, TX).

Xenopus oocyte injection. *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI) or *Xenopus* One (Beverly Hills, Mature females were anesthetized by immersion in a 0.15 to 0.2% tricaine methanesulfonate solution and oocytes were surgically removed. The follicular cell layer was partially removed after incubation for 2 to 3 hr. in a solution containing (in mM): NaCl (100), KCl (2), $MgCl_2$ (1), HEPES (5) and 1.5 mg/ml collagenase A. Defollicu tion was completed manually in most cases. Oocytes were inject with 10 to 50 nl containing 10 to 100 ng of combinations of hnAChR subunits $\alpha_X + \beta_X$ *in vitro* synthesized RNA. Pair-wise subunit combinations were injected at a 1:1 ratio. After injection, oocytes were incubated at 19°C for 3 to 5 days in a solution containing (in mM, NaCl (77.5), KCl (2), $CaCl_2$ (1.8), $MgCl_2$ (1), HEPES (5), Na-Pyruvate (5), with penicillin/streptomycin (10 ml/liter).

Drugs. ACh, NIC, CYT, DMPP, d-Tubo, MEC, atropine and collagenase A were purchased from Sigma Chemical Co. (St. Louis, MO). DHβE was purchased from Research Biochemicals International (RBI, Natick, MA). Stock solutions of agonists and antagonists were prepared and frozen. Individual aliquots were thawed and diluted in standard Ringer at the desired final concentrations.

Recording procedures. Oocytes were examined for functional expression 2 to 5 days after RNA injection using a two-electrode voltage-clamp protocol, with a GeneClamp 500 (Axon Instruments, Foster City, CA), or an Oocyte Clamp OC-725B (Warner Instrument Corp., Hamden, CT) amplifier. Axotape and pCLAMP software (Axon Instruments), Origin (Microcal, Northampton, MA) and Prism (Graphpad, San Diego, CA) software were used for data acquisition and analysis. Membrane potential was held at either -80 mV (for partial agonist DRCs and for antagonist experiments) or -60 mV (for full agonist DRCs); experiments were performed at room temperature (19–23°C). Microelectrodes were filled with a 3 M KCl solution (2–4 MΩ resistance). The extracellular recording solution (standard Ringer's) contained (in mM): NaCl (115), KCl (2.5), $CaCl_2$ (1.8), HEPES (10), atropine (0.001), pH 7.3. Perfusion solutions were gravity fed into the recording chamber (Warner Instruments, capacity: 110 μl) at a rate of 10 to 13 ml/min and were extruded at the opposite side of the chamber by vacuum; perfusate exchange was performed manually by switching between solenoid valves/reservoirs. Under these conditions, saturating concentrations of agonists could routinely activate currents with 0 to 100% rise-times of <200 msec, *e.g.*, response in hα7 in figure 1. Agonists were applied for approximately 10 sec in most experiments, although shorter (~5 sec) or longer (~20 sec) applications were also tested. Peak response amplitudes were measured and used in the determination of agonist and antagonist properties. Oocytes were washed in drug-free Ringer's for 3 to 10 min between successive drug applications for agonist and antagonist studies, except where otherwise indicated.

Agonist DRCs were obtained using two different methods. 1) For partial DRCs, responses were normalized to 1 μM ACh in all hnAChR subunit combinations, except hα7, normalized to 10 μM ACh. The normalizing dose of ACh was applied several times to each oocyte during the course of an experiment to check for desensitization; data were rejected if responses to the normalizing dose fell below 80% of the original response. 2) For full agonist DRCs, responses from each oocyte were normalized to the maximal response for each agonist tested and used to generate EC_{50} and n_H estimates. For comparison of relative agonist efficacies, the agonist responses for each oocyte were normalized to the response elicited by an EC_{80} dose of ACh (EC_{20} for hα3β2). For full DRCs, hnAChR subunit combinations containing $\beta 4$ subunits (hα2β4, hα3β4 and hα4β4) and hα7 were tested in a Ringers solution containing 0.18 mM [Ca^{2+}], to reduce the contribution of Ca^{2+}-activated Cl^- currents (Miledi and Parker, 1984) in agonist-induced responses. In this low [Ca^{2+}] Ringers solution, $\beta 2$-containing hnAChRs showed very small agonist-induced currents (≤40 nA to 1–3 mM ACh) and sensitivity to external Ca^{2+} [illegible] Mahaffy

The sensitivity to the nAChR antagonists d-Tubo, DHβE and MEC was tested in standard Ringer's solution. Each oocyte was tested at all concentrations indicated for the d-Tubo and DHβE experiments, except for d-Tubo on hα2β2 and hα3β4 nAChRs. For the latter, a different group of oocytes was tested with each antagonist dose, and one curve was fitted to the (averaged) data points. The activity of MEC was assessed at one dose (3 μM), due to the incomplete reversibility of the block by this antagonist.

Data analysis. Dose-response curves for agonists (full DRCs) and antagonists (d-Tubo and DHβE) were fitted by nonlinear regression to the equations: $I = I_{max}/(1 + (EC_{50}/Ag)^n]$ or $I = I_{max} - I_{max}/(1 + (IC_{50}/An)^n]$ wherein I_{max} = maximal normalized current response (in the absence of antagonist for inhibitory curves), Ag = agonist concentration, An = antagonist concentration, EC_{50} = agonist concentration eliciting half maximal current, IC_{50} = antagonist concentration eliciting half maximal current, and n = Hill coefficient. Antagonist curves were constrained to I_{max}=1 and I_{min}= 0. For agonist efficacy curves, I_{min} was constrained to 0, but I_{max} was not constrained.

Concentration data (EC_{50} and IC_{50} estimates) are shown as the geometric means ± S.D. Hill coefficient and efficacy estimates are shown as the arithmetic mean ± S.D. For the antagonists, IC_{50} values were converted to K_b values using the Leff-Dougall (Leff and Dougall, 1993) variant of the Cheng-Prusoff equation: $K_b = IC_{50}/((2 + ([Ag]/[A_{50}])^n)^{1/n} - 1)$, where Ag is the agonist, A_{50} is the EC_{50} value for the agonist and n = Hill coefficient.

Statistical tests. The geometric values for the EC_{50} or K_b data were tested for significant differences between receptor subtypes using a one-way analysis of variance followed by a Student-Newman-Keuls or Dunn's test for pairwise multiple comparisons. The Student-Newman-Keuls and the Dunn's tests (SigmaStat, ver. 1.01, Jandel Corporation, San Rafael, CA) provide a significance level of $P < .05$, but do not provide the absolute P value; therefore the differences may be of greater significance than stated in the text and tables. Differences in arithmetic n_H values for a given agonist between a β4- and a β2-containing hnAChRs, or differences in geometric IC_{50} values between DHβE and d-Tubo for each subunit combination were tested for significance with an unpaired two-tailed t test. The significance of differences in agonist potency from partial DRCs (or the potency of MEC) among hnAChRs subtypes, was tested with the Kruskal-Wallis one-way analysis of variance; followed by pairwise multiple comparisons with the Dunn's test (SigmaStat, ver. 1.01, Jandel Corporation).

Results

Human recombinant nAChRs display differential sensitivities to nicotinic agonists. The nicotinic agonists

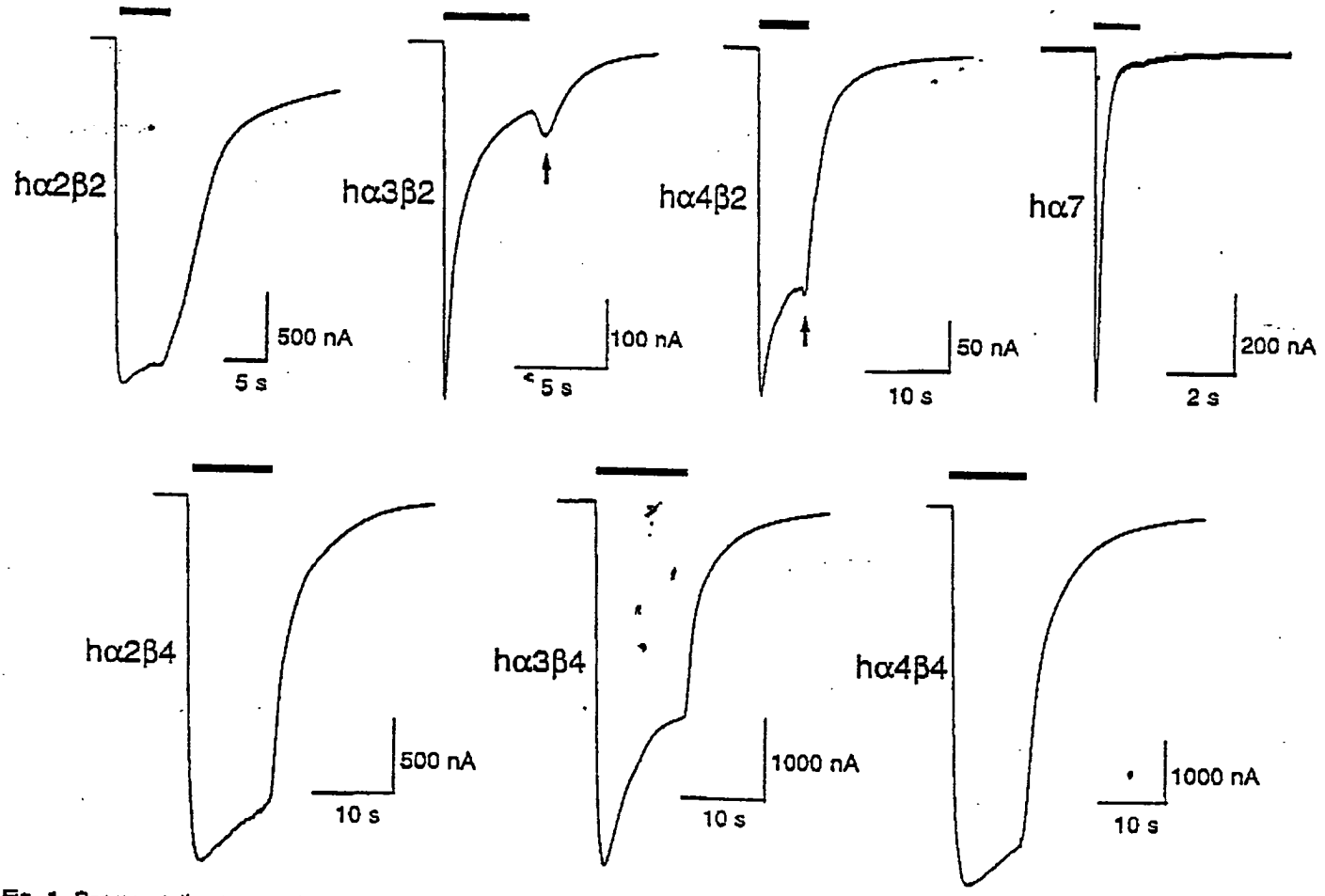

Fig. 1. Representative traces showing the current responses to maximally effective concentrations of ACh in oocytes injected with mRNA encoding various human nicotinic receptors. Data shown in figures 1 to 3 were obtained from oocytes voltage-clamped at -60 mV. Of the β2-containing receptors, hα3β2 receptors showed the fastest decay kinetics to ACh application. Similarly, hα3β4 receptors showed more apparent desensitization than did hα2β4 or hα4β4 receptors (bottom row). Currents recorded from hα7 nAChRs decayed very rapidly (upper right panel). Note the transient inward current observed in hα3β2- and hα4β2-injected oocytes upon removal of agonist (arrows). Maximally effective concentrations of ACh for the oocytes shown here were 300 μM for hα2β4 and hα4β4 receptors, 1 mM for hα2β2, hα3β4, hα4β2 and hα7 receptors and 3 mM for hα3β2 receptors

ACh, NIC, CYT and DMPP produced dose-dependent inward currents in voltage-clamped oocytes expressing different hnAChRs subunit combinations. The kinetics of agonist-induced currents were found to differ among the various subunit combinations (figs. 1 and 2A). Of the heteromeric nAChRs, currents generally decayed most rapidly in hα3β2 nAChRs; in contrast, currents elicited in hα2β2, hα2β4 and hα4β4 showed relatively little desensitization in the continued presence of high concentrations of agonists (fig. 1). Currents recorded from hα3β4 decayed substantially faster than those recorded in hα2β4 or hα4β4 nAChRs (figs. 1 and 2A). Responses from hα7 nAChRs decayed much more rapidly than those from any of the heteromeric nAChR subunit combinations (fig. 1). Agonist-dependent differences in the decay rate were also observed (for example in hα3β2: fig. 2, left panels), where a markedly faster decay rate was observed with DMPP than with CYT.

Human nAChRs subunit combinations exhibited distinct sensitivities to nicotinic agonists. Full dose response curves obtained for ACh, NIC, DMPP and CYT are shown in figure 3; data are summarized in table 1. The rank order of potency (EC_{50} estimates) derived from the full dose-response curves was the following (>indicates the significance level is $P < .05$ or higher, see "Methods"): hα2β2: DMPP~NIC~CYT~ACh; DMPP > ACh; hα2β4: NIC~DMPP > CYT>ACh; hα3β2: DMPP~CYT~NIC>ACh; hα3β4: DMPP > CYT~NIC>ACh; hα4β2: CYT>NIC>DMPP > ACh; hα4β4: CYT>NIC> DMPP~ACh and hα7: DMPP > CYT~NIC~ACh. These results show that ACh is the least potent of all agonists tested in most subunit combinations (hα2β4, hα3β2, hα3β4 and hα4β2).

Steeper agonist dose-response curves (fig. 3) and thus higher Hill coefficient values (table 1) were apparent in β4-containing hnAChRs, compared to β2-containing receptors coexpressed with the same α subunit. The differences in the Hill coefficients between hα2β2 and hα2β4 nAChRs for ACh, NIC and DMPP were significant ($P < .05$). Hill coefficient values were significantly larger for ACh, DMPP and CYT in hα3β4, compared to hα3β2 nAChRs ($P < .05$). Hill coefficient estimates from hα4β4 nAChRs were also significantly larger than those from hα4β2 for ACh, DMPP and CYT ($P < .05$). The large Hill coefficient for CYT on hα2β2 nAChRs is likely due to the low efficacy of the agonist on this subtype, which gives the smallest maximal responses.

Marked subtype-specific differences were also apparent in the relative efficacies displayed by these different nAChR agonists. CYT was least efficacious at β2-containing hnAChRs (hα2β2, hα3β2 and hα4β2), in contrast to its efficacy shown on β4-containing hnAChRs, hα2β4, hα3β4 and hα4β4. CYT displayed full agonist activity only at hα7 (fig. 3; table 1). ACh was the most or among the most efficacious agonists at all hnAChR subunit combinations except on hα3β2 hnAChRs, where DMPP was markedly more efficacious than ACh.

A dose-dependent increase in the rate of decay of agonist-induced inward currents was observed in all subunit combinations; this appeared more pronounced in hα3β2, hα3β4 (fig. 2) and hα7 nAChRs. This increase in the apparent rate of desensitization was also accompanied by a "rebound" inward current upon the removal of high doses of some agonists in some hnAChRs (figs. 1 and 2, arrows). This rebound current has been shown to be an indication of agonist-dependent open-channel block in native (Maconochie and Knight, 1992) and recombinant (Bertrand *et al*, 1992a) neuronal nAChRs. Long-lasting nAChR desensitization is supported by the observation that in some oocytes where an EC_{50} or EC_{20} dose of ACh was tested both before and up to 15 min after the completion of a full agonist DRC, the current amplitude to the second ACh application was reduced. This was more evident on hα3β2-expressing nAChRs, but was also observed in β4-containing hnAChRs. This long-lasting form of desensitization was not observed in oocytes expressing hα7 nAChRs.

These observations indicate that application of mid to high

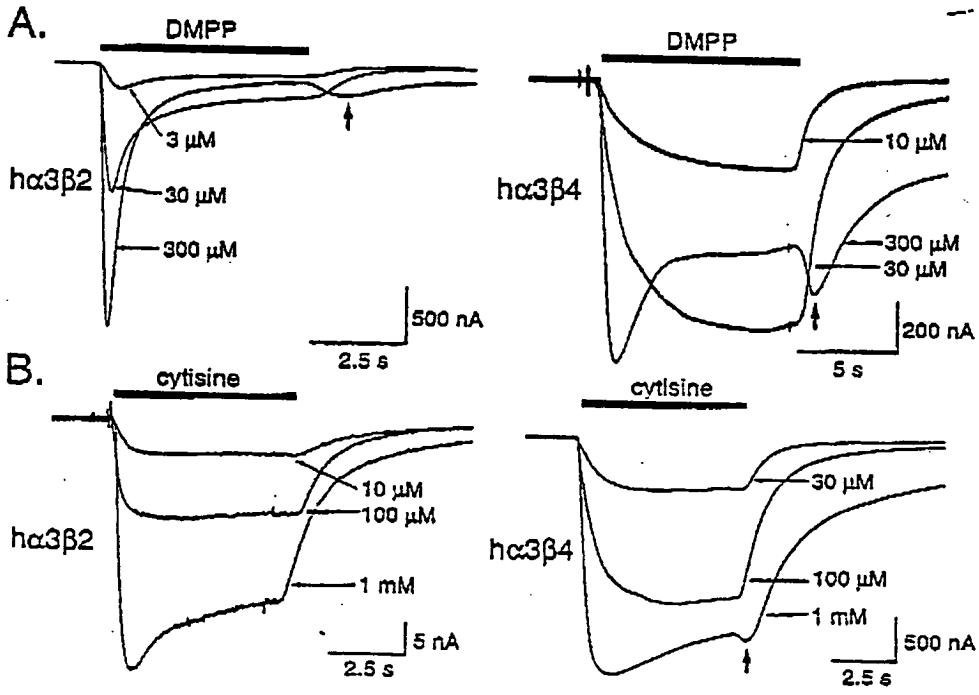

Fig. 2. Dose-dependent responses elicited by the application of the nicotinic agonists DMPP and CYT in oocytes expressing the hnAChRs subunit combinations hα3β2 or hα3β4. A, DMPP produced rapidly decaying responses in an oocyte injected with hα3β2 mRNA (left) but more slowly decaying responses in an hα3β4-injected oocyte (right). In each of these oocytes, application of a high agonist concentration produced a rapidly decaying response followed by a transient inward current when switching from DMPP-containing to control medium (arrows). B, Current responses elicited by the application of various concentrations of CYT showed similar decay properties in oocytes expressing hα3β2 or hα3β4 nAChRs. As with DMPP, switching from cytisine-containing to control medium produced a small inward current not seen at lower agonist concentrations. A through D represent data obtained from four different oocytes.

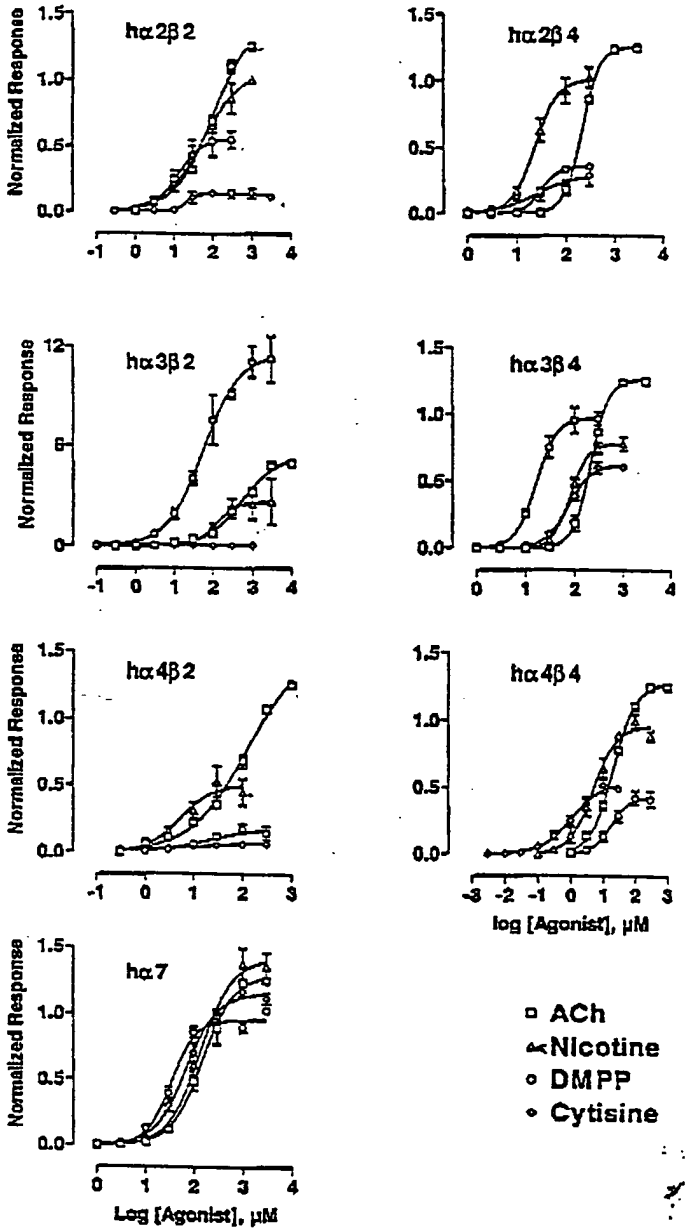

Fig. 3. Full dose-response curves for ACh, (-) NIC, DMPP and CYT on recombinant hnAChRs. Current responses in each oocyte were normalized to the EC_{80} (or EC_{20} for hα3β2) ACh response recorded in the same oocyte. Data points indicate the mean ± S.E.M. of three to six oocytes. Where no error bars are seen, they are smaller than the symbols.

agonist concentrations, such as those required to achieve saturation of agonist DRCs, can result in desensitization and/or agonist-induced channel block of neuronal nAChRs. Both can contaminate efficacy, potency and Hill coefficient values; therefore, these estimates may not directly reflect the interaction of the ligand with the nAChRs.

To address this issue, we have compared the rank order of potencies estimated from full dose-response curves with those obtained in a separate series of experiments from partial dose-response curves (fig. 4), similar to those reported for recombinant rat nAChRs (Luetje and Patrick, 1991; Connolly *et al.*, 1992; Covernton *et al.*, 1994). These experiments were designed to test the relative sensitivity of hnAChRs at agonist concentration ranges where desensitization would be expected to be small (0.3 to 10–30 μM). They also serve as a comparison to the only other study that has compared the agonist profiles among all six pair-wise nAChRs subunit combinations using the rat homologs (Luetje and Patrick, 1991). Data were rejected if responses to the normalizing dose fell below 80% of the initial response (see "Methods"). We found that the relative potency displayed by these four agonists, in the ranges ≤ 30 μM, was similar using both methods for all hnAChRs except hα2β4. In hα2β4 the relative potency of DMPP, NIC and ACh appeared different: NIC≈ACh>DMPP with partial DRCs (fig. 4), whereas NIC>DMPP > ACh was observed at 10–30 μM in the full DRCs (fig. 3). Using either method, CYT elicited the largest responses at doses ≤3 μM in this subunit combination. From the partial DRCs, it is apparent that CYT is the least potent agonist at β2-containing hnAChRs, whereas it is the most potent agonist at hα2β4 and hα4β4 nAChRs. These results are similar to what has been reported for their rat homologs and are consistent with the idea that β subunits also contribute to the pharmacology of neuronal nAChRs (Luetje and Patrick, 1991).

To analyze the effect of agonist-induced nAChR desensitization and/or channel block on the Hill coefficient estimates obtained from the fits to the full DRCs, we examined the slope of log-log plots from the partial agonist DRCs. The slope of log-log plots of DRCs at low agonist concentration ranges approximates the Hill coefficient (cf. Connolly *et al.*, 1992; Covernton *et al.*, 1994; Cohen *et al.*, 1995). Using low concentrations of ACh (≤30 μM) to minimize nAChR desensitization and the contribution of the endogenous Ca^{2+}-activated Cl^- current, we have compared the slopes of these dose-response log-log plots among the different hnAChR subtypes. The slopes of the ACh log-log plots were markedly steeper for β4-containing hnAChRs (and hα7) than for β2-containing hnAChRs (fig. 5). Log-log plots obtained for the other agonists also displayed shallower slopes for nAChRs containing the β2 subunit than those containing β4 subunits (data not shown). The differences observed in log-log DRC slopes between β2- and β4-containing hnAChRs are in agreement with the results obtained with the n_H estimates derived from the full DRCs.

Recombinant hnAChRs show a unique sensitivity to nAChRs blockers. We have tested the sensitivity of these recombinant hnAChRs to the nAChR antagonists d-Tubo, DHβE and MEC. Dose-response curves for d-Tubo and DHβE inhibition were constructed for each hnAChR subunit combination; sensitivity to MEC was tested at a single concentration (3 μM; see "Methods"). The agonist and dose to test these antagonists on each subunit combination were selected on the basis of 1) potency: the most or one of the most potent agonist was used and, 2) magnitude of the response: a concentration eliciting a large response, but relatively small desensitization upon repeated application (see fig. 4). The agonists and doses selected were the following: hα2β2: 10 μM NIC; hα2β4: 30 μM ACh; hα3β2: 10 μM DMPP; hα3β4: 10 μM DMPP; hα4β2: 10 μM ACh; hα4β4: 10 μM NIC; hα7: 100 μM ACh.

DHβE and d-Tubo reversibly inhibited agonist-induced currents in oocytes expressing these different hnAChRs (fig. 6). The reversibility of nicotinic responses after MEC appli-

TABLE 1

Comparison of potency and efficacy of nAChRs agonists on recombinant hnAChRs*

Agonist	hα2β2	hα2β4	hα3β2	hα3β4	hα4β2	hα4β4	hα7
ACh (*N*)	(4)	(4)	(5)	(4)	(4)	(4)	(5)
EC_{50}	68.67 μM	82.57 μM	442.90 μM	203.14 μM	68.05 μM	19.68 μM	179.57 μM
(−S.D., +S.D.)	(61.56, 76.61)	(71.61, 95.21)	(298.78, 656.55)	(191.15, 215.89)	(54.15, 85.51)	(16.45, 23.53)	(132.08, 244.17)
n_H	1.22 ± 0.1	1.70 ± 0.1	1.17 ± 0.1	2.24 ± 0.2	1.02 ± 0.04	1.28 ± 0.04	1.46 ± 0.02
I_{max}	1.25	1.25	5.0	1.25	1.25	1.25	1.25
NIC (*N*)	(6)	(4)	(6)	(4)	(5)	(4)	(4)
EC_{50}	19.23 μM	20.71 μM	132.44 μM	80.30 μM	5.47 μM	5.02 μM	113.34 μM
(−S.D., +S.D.)	(15.64, 23.65)	(19.34, 22.19)	(67.23, 260.89)	(78.20, 82.47)	(4.48, 6.68)	(2.53, 9.97)	(85.84, 195.11)
n_H	1.38 ± 0.1	2.24 ± 0.1	2.54 ± 0.7	2.38 ± 0.1	1.26 ± 0.05	1.28 ± 0.1	1.61 ± 0.1
I_{max}	0.77 ± 0.11	1.03 ± 0.07	2.84 ± 0.52	0.78 ± 0.05	0.52 ± 0.12	1.0 ± 0.05	1.38 ± 0.11
DMPP (*N*)	(4)	(4)	(3)	(4)	(4)	(4)	(4)
EC_{50}	11.18 μM	22.77 μM	55.87 μM	18.67 μM	17.99 μM	18.71 μM	30.86 μM
(−S.D., +S.D.)	(8.48, 14.74)	(18.47, 28.06)	(44.02, 70.93)	(15.60, 22.36)	(17.15, 18.86)	(11.70, 29.93)	(3.15, 41.14)
n_H	1.44 ± 0.1	2.16 ± 0.2	0.97 ± 0.1	1.93 ± 0.3	1.24 ± 0.05	1.56 ± 0.1	2.18 ± 0.4
I_{max}	0.53 ± 0.02	0.36 ± 0.02	11.20 ± 1.42	0.98 ± 0.07	0.14 ± 0.05	0.42 ± 0.05	1.02 ± 0.01
CYT (*N*)	(4)	(4)	(3)	(4)	(5)	(4)	(4)
EC_{50}	25.43 μM	38.86 μM	67.09 μM	72.18 μM	2.61 μM	0.80 μM	71.42 μM
(−S.D., +S.D.)	(13.78, 46.92)	(31.15, 48.47)	(51.12, 88.04)	(64.77, 80.44)	(1.69, 4.05)	(0.84, 0.97)	(45.56, 111.96)
n_H	13.37 ± 5.04	1.01 ± 0.01	0.95 ± 0.04	1.91 ± 0.1	0.61 ± 0.06	1.0 ± 0.04	1.41 ± 0.03
I_{max}	0.13 ± 0.02	0.29 ± 0.08	0.038 ± 0.003	0.60 ± 0.04	0.06 ± 0.01	0.51 ± 0.06	1.16 ± 0.03

* Potency (EC_{50}) expressed as geometric mean, Hill coefficient (n_H) and efficacy (I_{max}) are expressed as the arithmetic mean ± S.E.M. *N* indicates the number of oocytes included in the estimates and I_{max} represents the fraction of the ACh EC_{50} response (EC_{20} for hα3β2).

cation (3 μM) was variable. In some cells, full recovery was not observed after prolonged (10–15 min) washout in drug-free Ringer's. A differential sensitivity to the three antagonists was observed (figs. 7–9). Table 2 summarizes the K_b estimates obtained from the Leff-Dougall variant of the Cheng-Prusoff equation (Leff and Dougall, 1993), which corrects for both the potency of the agonist used and its Hill coefficient from the agonist DRCs. The K_b estimates for DHβE and d-Tubo from the DRCs (fig. 7) indicate that hα4β2 and hα4β4 nAChRs are more sensitive to block by DHβE than d-Tubo ($P < .01$, *t* test), whereas hα7 ($P < .01$, *t* test) and hα3β4 are more sensitive to block by d-Tubo than DHβE. In contrast, no significant difference in the K_b estimates for these two antagonists was found in hα2β2, hα2β4 and hα3β2 nAChRs ($P > .05$). Human α4β4 was the nAChR subtype most sensitive to block by DHβE and d-Tubo. K_b values for d-Tubo were significantly lower for hα4β4 than those of hα4β2, hα2β4 and hα7 ($P < .05$). The rank order of potency of DHβE was hα4β4>hα4β2>hα2β2≈hα3β2≈hα2β4>hα3β4≈hα7 (> indicates the significance level is $P < .05$).

The effect of d-Tubo appeared unusual on some hnAChRs. The inhibition by this antagonist on hα2β4 nAChRs was more dramatic at later times after the activation of the inward current than at the initial peak (fig. 8). This effect, observed in all six cells tested, was noticeable at concentrations of d-Tubo of 0.3 μM and above. The effect on the kinetics of agonist-induced responses produced by d-Tubo is similar to that produced by MEC, but different from the effect of DHβE on this and other hnAChR subunit combinations tested. Our observations suggest that d-Tubo may act noncompetitively at hα2β4 nAChRs, in addition to its putative action at the ligand binding site. d-Tubo also appeared to alter the kinetics of agonist-induced responses on hα4β4, but not hα2β2 nAChRs (data not shown).

MEC (3 μM) inhibited agonist-induced responses by >80% in hα2β4 and hα4β4 and by ~50% in hα2β2, hα4β2 and hα7 nAChRs (fig. 9). The sensitivity to MEC observed in hα4β4 nAChRs was significantly more than that observed in hα2β2, hα4β2 or hα7 hnAChRs ($P < .05$ Dunn's test).

Discussion

We have shown that recombinant hnAChRs display differential sensitivities to nicotinic agonists and antagonists, and that both α and β subunits contribute to the pharmacology of these ligand-gated channels.

Agonist selectivity of recombinant hnAChRs. Full agonist DRCs were obtained for ACh, NIC, DMPP and CYT. Our results indicate that when high agonist concentrations are used, such as those required to reach saturation in DRCs, receptor activation can overlap with agonist-induced receptor desensitization and/or channel block, which can contaminate efficacy, slope and potency estimates. These phenomena are not unique to nAChRs (Luetje and Patrick, 1991; Connolly *et al.*, 1992; Maconochie and Knight, 1992), but are also observed in other ligand-gated channels (for review, see Jones and Westbrook, 1996). Results derived from full DRCs were therefore compared with those obtained from partial DRCs. To our knowledge, this is the first study in which the agonist pharmacology of recombinant nAChRs is evaluated with both partial DRCs (in which agonist concentrations tested are low to minimize receptor desensitization) and full DRCs. At low agonist concentrations, no differences in relative agonist potency were noted between full and partial DRCs in any of the hnAChRs, except for hα2β4. Also, the relatively larger n_H estimates observed in β4- compared to their related β2-containing hnAChRs were observed both in partial DRCs and full DRCs.

An interesting observation is the evaluation of the differential activity of CYT on β2 *vs.* β4-containing hnAChRs. Fully saturating DRCs were obtained for CYT in all of the β2-containing receptors, albeit with very low efficacy, yielding actual EC_{50}, n_H and I_{max} values (see table 1 and fig. 2). The EC_{50}s from these determinations showed similar or

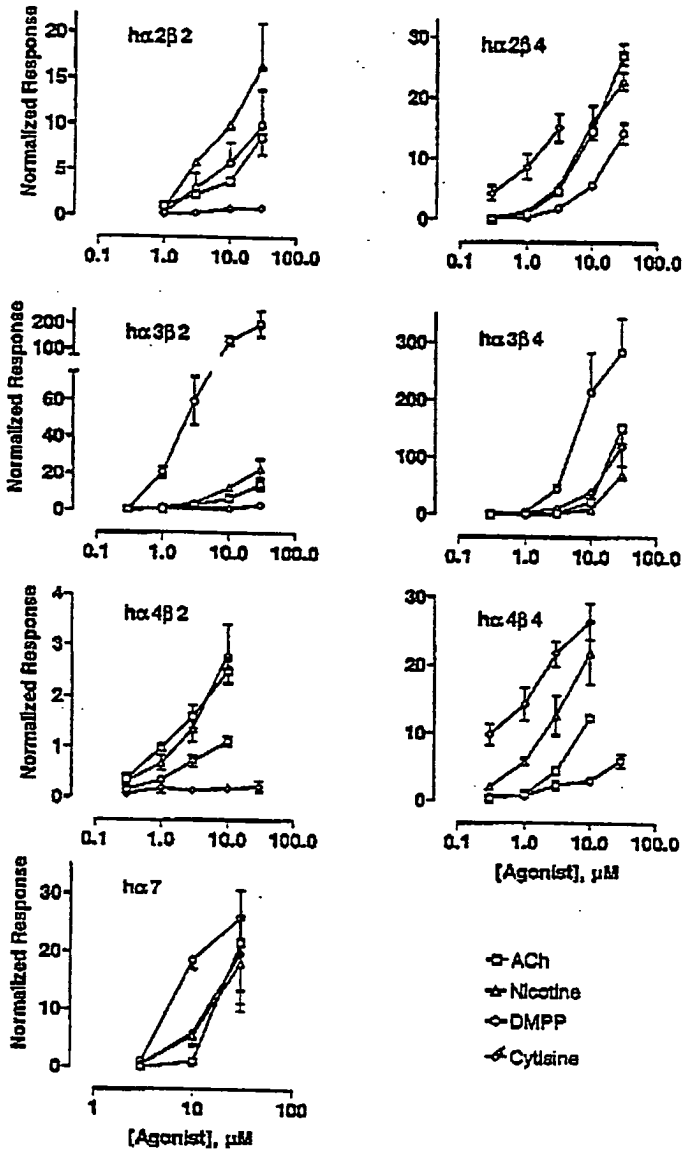

Fig. 4. Partial dose-response curves for hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 nAChRs. Responses to the agonists ACh, (-) NIC, DMPP and CYT, were normalized to the amplitude of the response elicited by 1 μM ACh in the same oocyte, except for hα7, where responses were normalized to 10 μM ACh (response amplitude elicited by 1 or 10 μM ACh = 1). Each symbol represents the mean ± S.E.M. of the responses observed in 3 to 12 oocytes. Where no error bars are seen, they are smaller than the symbols.

higher potency than the other agonists examined on β2-containing receptors. However, when partial DRCs were constructed, CYT was seen to have a very low potency relative to other agonists at equivalent concentrations (fig. 4). This latter observation is similar to that reported for the rat homologs (Luetje and Patrick, 1991; Covernton *et al.*, 1994) and can be overlooked when the sensitivity to agonists is evaluated from full DRCs.

A marked difference was observed in the kinetics of currents elicited in hα3β4 and hα3β2 nAChRs, with currents elicited on hα3β2 nAChRs decaying more rapidly than those recorded in hα3β4 nAChRs. This is in agreement with the kinetics reported for responses to epibatidine on these

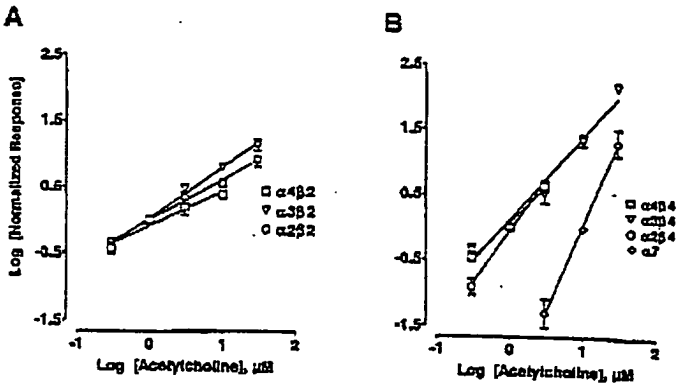

Fig. 5. Log-log plot of the ACh dose-response relation for (A) β2-containing hnAChRs and (B) β4-containing and hα7 hnAChRs. Data points represent the mean ± S.E.M. of the responses normalized to the current elicited by 1 μM ACh in each oocyte (n = 3–10 oocytes). Regression lines were fitted using least squares approximation to the data points. The slope values of these lines are 0.62 for hα2β2, 0.79 for hα3β2, 0.53 for hα4β2, 1.61 for hα2β4, 1.26 for hα3β4, 1.11 for hα4β4 and 2.08 for hα7. Where no error bars are seen, they are smaller than the symbols.

hnAChRs (Gerzanich *et al.*, 1995) and for agonist-induced currents in rat α3β2 and α3β4 (Cachelin and Jaggi, 1991; Cohen *et al.*, 1995). The fast kinetics of agonist-induced currents observed in hα7 nAChRs are not different from those reported by Peng *et al.* (1994) and Gopalakrishnan *et al.* (1995) for hα7 and for rat (Séguéla *et al.*, 1993) and chick α7 (Couturier *et al.*, 1990).

The agonist selectivity profile of hα3β2, hα3β4 and hα7 nAChRs reported differs from that reported for their rat homologs. DMPP is more potent than ACh in hα3β2 nAChRs (figs. 3 and 4), whereas the rank order of potency reported for rat α3β2 is DMPP=ACh>NIC>CYT (Luetje and Patrick, 1991). However, two groups have reported that DMPP > ACh for rat α3β2 nAChRs expressed in *Xenopus* oocytes (Cachelin and Jaggi, 1991; Covernton *et al.*, 1994); the reason for this discrepancy is unclear. NIC is more potent than ACh at hα3β2 nAChRs (table 1), in agreement with the recently reported rank order of potency of epibatidine>NIC>ACh for hα3β2 nAChRs expressed in *Xenopus* oocytes (Gerzanich *et al.*, 1995), but different from the profile reported for the rat α3β2 (ACh>NIC: Luetje and Patrick, 1991; Covernton *et al.*, 1994). Although the rank order of potencies for ACh and NIC agree between our work and that of Gerzanich *et al.* (1995), the EC_{50} estimates obtained for ACh and NIC do not. Higher values were observed in this study, compared to those of Gerzanich *et al.* (1995). However, Gerzanich *et al.* used the pSP64T vector for expression of hα3β2 and hα3β4. We examined the potency of ACh and NIC with hβ2 cDNA subcloned into the pSP64T vector and observed ACh and NIC EC_{50}s of 1.75 ± 0.1 μM (n=3) and 0.79 ± 0.22 μM (n=3) for hα2β2, 27.4 ± 8.1 μM (n=4) and 21.1 ± 3.4 μM (n=4) for hα3β2 and 1.3 ± 0.1 μM (n=3) and 0.3 ± 0.1 μM (n=3) for hα4β2. The values that we observed for hα3β2 using the pSP64T vector are similar to those observed by Gerzanich *et al.* for hα3β2. The reason for the differences with these vectors is not understood. By contrast, we did not observe potency differences for ACh or NIC with hα3β4 using hβ4 cDNA (KEβ4.6) subcloned into the pCMV-T7 vector (table 1) compared to the results observed by Gerzanich *et al.* using the pSP64T vector.

DMPP is the most potent agonist at hα3β4 nAChRs (figs. 3

hα2β2 displays a similar sensitivity to these two antagonists, larger than that of hα3β2, and this difference is statistically

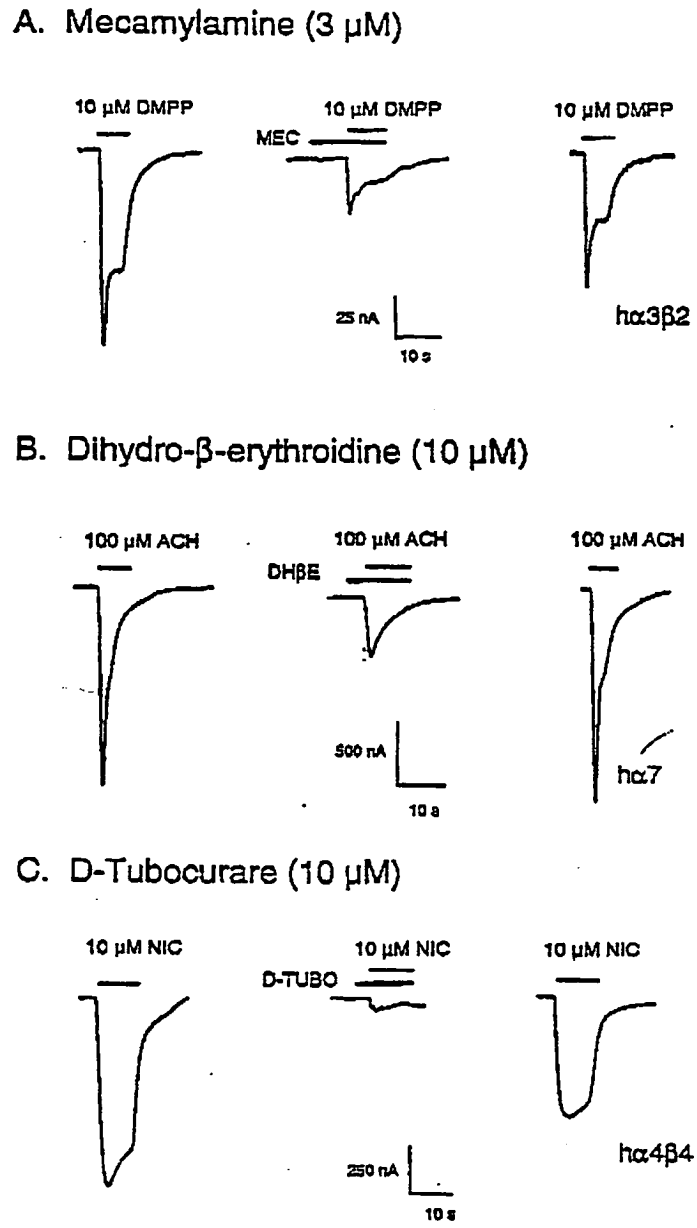

Fig. 6. Inhibition of agonist-induced currents by nicotinic receptor antagonists. Current responses recorded from oocytes expressing hα3β2 (A), hα7 (B) or hα4β4 (C) nAChRs. Traces shown on each row are from the same oocyte. The time between each application (control, agonist + antagonist and wash) was 5 to 10 min.

and 4). In contrast, the rank order of potency reported for rat α3β4 is CYT>NIC=ACh≥DMPP (Luetje and Patrick, 1991; Covernton *et al.*, 1994). NIC and ACh are also equipotent in rat α3β4 nAChRs transiently expressed in mammalian HEK-293 cells (Wong *et al.*, 1995), whereas NIC>ACh at hα3β4 nAChRs (Table 1), in agreement with the rank order of potency of epibatidine>NIC>ACh reported for hα3β4 (Gerzanich *et al.*, 1995). The relative efficacies reported for ACh, NIC, CYT and DMPP for rat α3β4 also differ from the efficacies found in this study (Wong *et al.*, 1995). Taken together, these data indicate that the pharmacology of hα3β4 and hα3β2 nAChRs differs from that of their rat homologs.

The agonist sensitivity observed in hα7 nAChRs is in agreement with [illegible] *Xenopus* oocytes by Peng *et al.* (1994), but it differs from the sensitivity reported for the rat (NIC>CYT>DMPP > ACh) (Séguéla *et al.*, 1993) and the chick homologs (NIC=CYT> ACh>DMPP) (Bertrand *et al.*, 1992b) in that DMPP is the most potent agonist at hα7 nAChRs. However, Gopalakrishnan *et al.* (1995) recently reported an agonist pharmacology for hα7 stably transfected in HEK-293 cells that is closer to that reported for the rat, wherein NIC is the most potent agonist. The reason for this discrepancy is not clear; the full cDNA sequence of Gopalakrishnan *et al.* (1995) for the hα7 clones used has not been published.

The rank order of potency observed for nAChR subunit combinations hα2β2, hα4β2 and hα4β4 is similar to that reported for their rat homologs (fig. 4) (Luetje and Patrick, 1991; Connolly *et al.*, 1992). Furthermore, the relative sensitivity to nicotinic agonists recently reported using a $^{86}Rb^{+}$ efflux assay in hα4β2 nAChRs stably expressed in HEK293 cells (Gopalakrishnan *et al.*, 1996) is in agreement with our results.

Interestingly, even the minor divergence found in the sequence of the amino terminal extracellular domain of α subunits between human and rat may contribute to the pharmacological differences observed between some recombinant hnAChRs and their rat homologs, because a single amino acid substitution in this region can profoundly affect the pharmacology of recombinant nAChRs (Hussy *et al.*, 1994; Galzi *et al.*, 1991). The identity between human and rat deduced amino acid sequences in this domain is 93% for α3 and 94% for α7 subunits (Elliott *et al.*, 1996). Our observations with hα3β2, hα3β4 and hα7 nAChRs suggest that the divergence in molecular structure between human and rat nAChR subunits α3 and α7 may account for the altered pharmacological properties of their assembled multimeric receptors.

The Hill slope values we obtained for some agonists in β2-containing hnAChRs are lower than those obtained for β4-containing hnAChRs. Lower Hill coefficients have been reported for nicotinic agonists in rat α3β2 compared to rat α3β4 nAChRs expressed in *Xenopus* oocytes (Cachelin and Jaggi, 1991; Covernton *et al.*, 1994; Cohen *et al.*, 1995). It is possible that nAChRs containing β2 subunits desensitize more rapidly than β4-containing receptors and that this desensitization accounts for the lower Hill coefficient estimates; however, typically faster decay rates were observed in hα3β2 nAChRs than in hα2β2 or hα4β2 nAChRs, and yet comparable Hill values were obtained in these subtypes. Alternatively, the differences in Hill coefficients may reflect different interactions between β2 and β4 subunits with α subunits, determining the cooperativity of the assembled receptors, as proposed by Cohen *et al.* (1995).

Sensitivity to block by nicotinic receptor antagonists. Recombinant hnAChRs are inhibited by the nicotinic receptor antagonists MEC, DHβE and d-Tubo, and distinct relative sensitivities to these antagonists were observed among the seven hnAChRs subunit combinations. Receptors containing α4 subunits, hα4β2 and hα4β4, were the only hnAChRs tested that display a higher sensitivity to DHβE than to d-Tubo. This result underscores the relevance of α subunits in determining the antagonist profile of these receptors. Human α4β2 and hα4β4 nAChRs can be differentiated by their sensitivity to MEC, with hα4β4 being more

TABLE 2

Inhibition of agonist-induced currents in recombinant hnAChRss by DHβE and d-Tubo

	hα2β2	hα2β4	hα3β2	hα3β4	hα4β2	hα4β4	hα7
DHβE[a]							
K_b mean [b]	0.85 μM	3.61 μM	1.62 μM	13.77 μM	0.11 μM	0.01 μM	19.59 μM
(−S.D., +S.D.)[b]	(0.78, 0.93)	(1.65, 7.90)	(0.89, 2.94)	(11.34, 16.73)	(0.09, 0.14)	(0.01, 0.04)	(11.1, 34.57)
n_H (mean ± S.E.M.)	0.94 ± 0.09	0.99 ± 0.12	1.33 ± 0.26	0.94 ± 0.09	0.58 ± 0.05	0.88 ± 0.10	1.12 ± 0.11
(N)	(3)	(4)	(5)	(4)	(4)	(5)	(3)
d-Tubo[a]							
K_b mean[b]	1.36 μM[c]	4.24 μM	2.41 μM	2.24 μM[c]	3.16 μM[d]	0.21 μM[d]	3.10 μM[d]
(−S.D., +S.D.)[b]		(1.98, 9.09)	(1.89, 3.09)		(2.43, 4.13)	(0.07, 0.64)	(1.67, 5.75)
n_H (mean ± S.E.M.)	0.68[c]	0.81 ± 0.09	1.09 ± 0.14	1.03[c]	0.74 ± 0.04	0.95 ± 0.11	1.69 ± 0.30
(N)	(5)	(6)	(5)	(4)	(4)	(3)	(6)

[a] The agonist and dose used for each hnAChRs subunit combination are indicated in the text.
[b] Geometric mean values (see "Methods").
[c] A different group of oocytes was tested with each antagonist dose, and therefore, only one curve was fitted to the (averaged) data points (see "Methods").
[d] The difference in potency between d-Tubo and DHβE was statistically significant ($P < .01$).

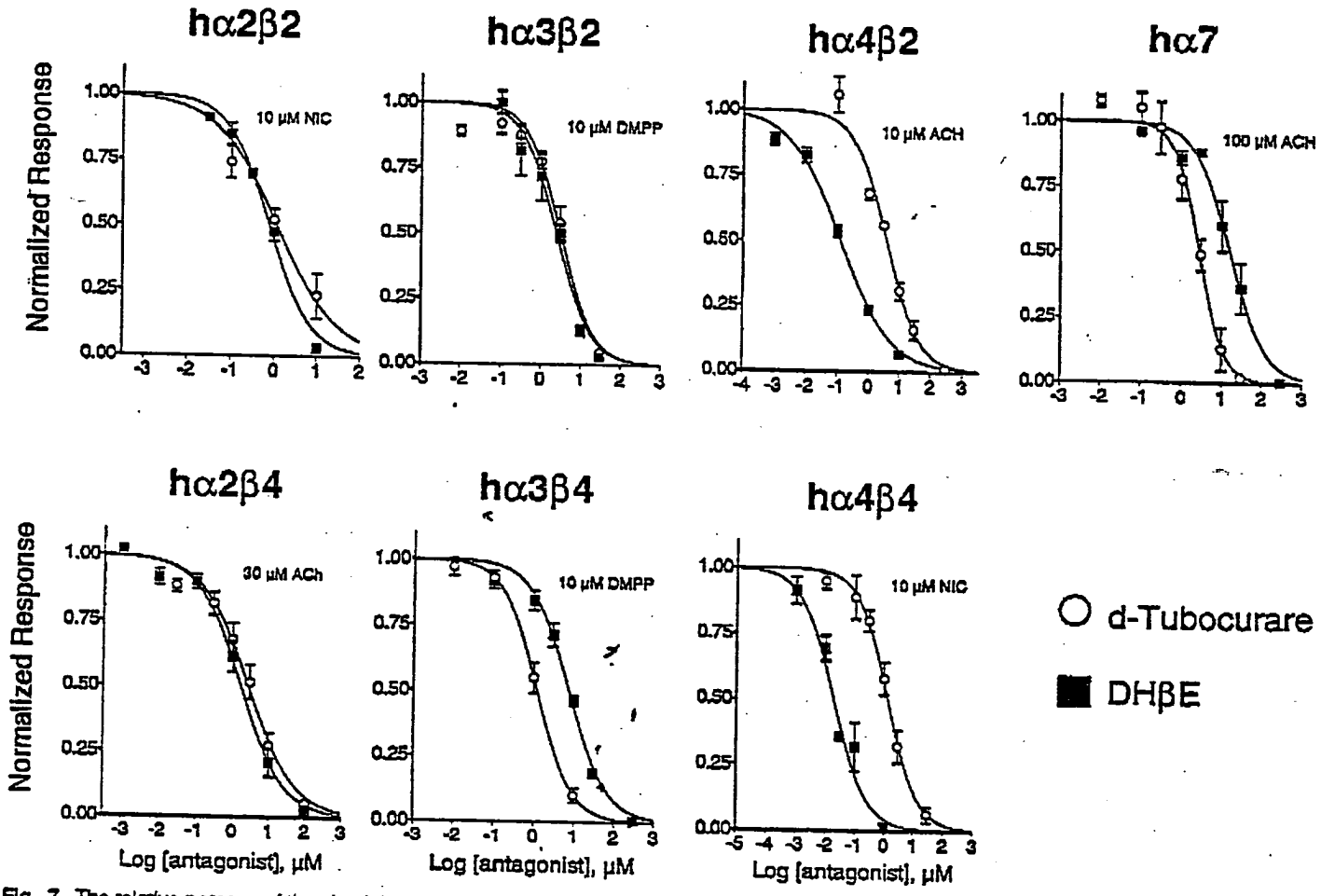

Fig. 7. The relative potency of the nicotinic receptor antagonists d-Tubo and DHβE differs among recombinant hnAChRs. Dose-response curves (fitted by nonlinear regression to the Hill equation, see "Methods") for d-Tubo and DHβE on all seven hnAChRs. Response amplitudes recorded upon the coapplication of either of these antagonists and a nicotinic agonist (indicated on the right of each plot), were normalized to the current amplitude elicited by the agonist alone. Data points represent the mean ± S.E.M. of the responses observed in three to six oocytes. The difference in potency between d-Tubo and DHβE was statistically significant for hα4β2, hα4β4 and hα7 ($P < .05$, Mann-Whitney); hα3β4 nAChRs cannot be tested for significance (see "Methods").

sensitive than hα4β2 nAChRs. Conversely, hα2β2, hα4β2 and hα7 nAChRs, which display a similar sensitivity to MEC, show different sensitivity profiles to DHβE and d-Tubo: hα2β2 displays a similar sensitivity to these two antagonists, hα4β2 is more sensitive to DHβE than d-Tubo, and hα7 is more sensitive to d-Tubo than DHβE.

Our K_b estimate for DHβE on hα3β4 nAChRs is ~9-fold larger than that of hα3β2, and this difference is statistically

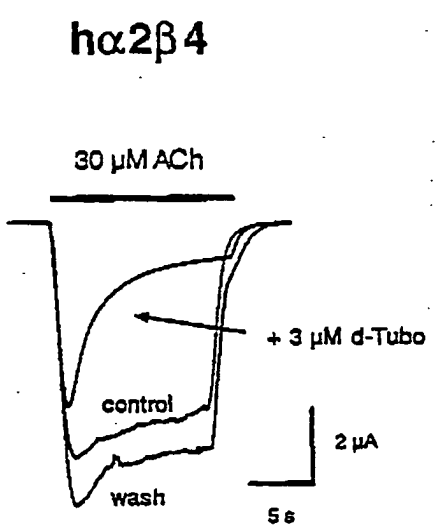

Fig. 8. The kinetics of agonist-induced currents in hα2β4 nAChRs are altered by coapplication with submaximal doses of d-Tubo. Current responses elicited in an oocyte expressing hα2β4 nAChRs by ACh in the absence (control), in the presence (arrow) and after washout of 3 μM d-Tubo (wash). Holding membrane potential -80 mV.

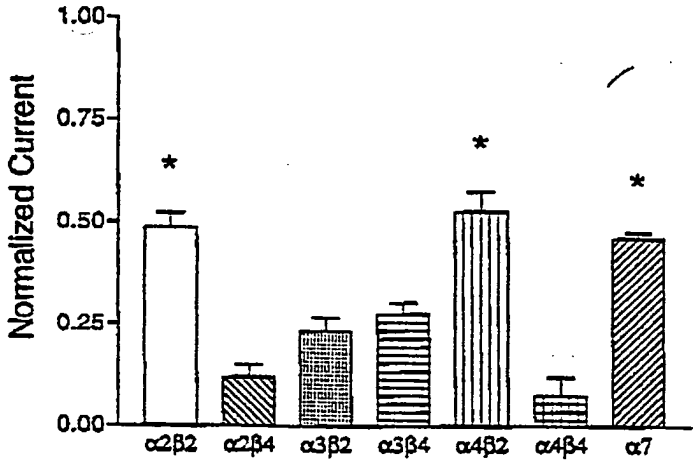

Fig. 9. Amplitude of the responses (mean ± S.E.M.) elicited by nicotinic agonists in the presence of 3 μM MEC as a fraction of the response recorded in its absence (n = 3–10 oocytes/group). The nicotinic agonist used for each nAChRs subunit combination is indicated in table 1. The sensitivity to MEC observed in hα4β4 is significantly different from that seen in hnAChR indicated by an asterisk ($P < .0001$, Kruskal-Wallis analysis of variance, followed by Dunn's test, $P < .05$,).

significant. These results agree with the higher sensitivity to DHβE reported for rat α3β2, compared with rat α3β4 nAChRs (Harvey and Luetje, 1996) and emphasize the importance of α/β subunit interactions in the determination of the pharmacological properties of nAChRs.

The activity of d-Tubo on hα2β4 and hα4β4 suggests that a noncompetitive block mechanism is also involved in the inhibition of agonist-induced currents. It is also possible, however, that slower binding kinetics of d-Tubo to the agonist recognition site, compared to that of the agonist, contribute to our observations. Further studies are required to determine the mechanism of action of d-Tubo on these and other hnAChRs subunit combinations. This putative noncompetitive action of d-Tubo may compromise the utility of the K_b transformation of the IC_{50} values. A noncompetitive antagonism by d-Tubo has been reported for recombinant chick α7 nAChRs expressed in *Xenopus* oocytes (Bertrand *et al.*, 1992b), and a voltage-dependent block by d-Tubo (but not DHβE) has been observed in native rat nAChRs (Mulle *et al.*, 1991).

The sensitivity to DHβE appears different between human (K_b = 19.6 μM) and chick α7 nAChRs (1.6 μM; 100 μM ACh as agonist, Bertrand *et al.*, 1992b). Our K_b estimate for hα7 of 3.10 μM for d-Tubo was somewhat higher than the IC_{50} value of 0.7 μM estimate reported by Peng *et al.* (1994) for hα7 nAChRs; this may partly be due to the lower agonist concentration used in their study (30 μM NIC). These IC_{50} values are comparable to the estimated IC_{50} of 0.55 μM reported for rat α7 (Seguela *et al.*, 1993). No large differences were found in the sensitivities to MEC between hα2β2 and hα4β2 and their rat homologs. An IC_{50} of about 3 μM for MEC may be estimated for these hnAChRs, which is comparable to the IC_{50} of 1 μM estimated for their rat homologs (Connolly *et al.*, 1992).

The pharmacological profile of the different hnAChR subunit combinations observed in this study may help in the determination of the molecular composition of native nAChRs involved in agonist-induced responses in human cells. In particular, the agonist profile reported for the SH-SY5Y human neuroblastoma cell line using the Rb^+ flux assay (Lukas *et al.*, 1993) indicates that hnAChRs containing α3β4 subunits significantly contribute to the functional nAChR pool in these cells.

Taken together, our data indicate that recombinant human nAChRs display unique pharmacological properties that are determined by their α and β subunits. Also, some pharmacological differences are apparent between human and rat (and chick) homologs. The distinct agonist/antagonist selectivity profiles observed for recombinant hnAChRs demonstrate the potential for discovery and development of subtype selective nicotinic ligands. Furthermore, the differences between the pharmacological properties of human and rat recombinant nAChRs underscores the importance of screening human nAChRs for the identification and development of nAChR ligands as potential therapeutic drugs.

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